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The Editor comments

MISPLACED CONFIDENCE

When the founding fathers of this great nation established it upon principles of a democratic form of government, they undoubtedly did so with a firm conviction that a people so empowered would have the good judgment to entrust matters of a technical nature to elected or appointed experts in the field. For the most part we have seen the American people follow this sensible approach. The highway engineers determine whether a new highway is needed, and if so, its location; the post office department decides upon a new post office building, its size, and its staff; the school board selects teaching personnel, and determines local policy with regard to standards of education; and so it is with most of our departments of government.

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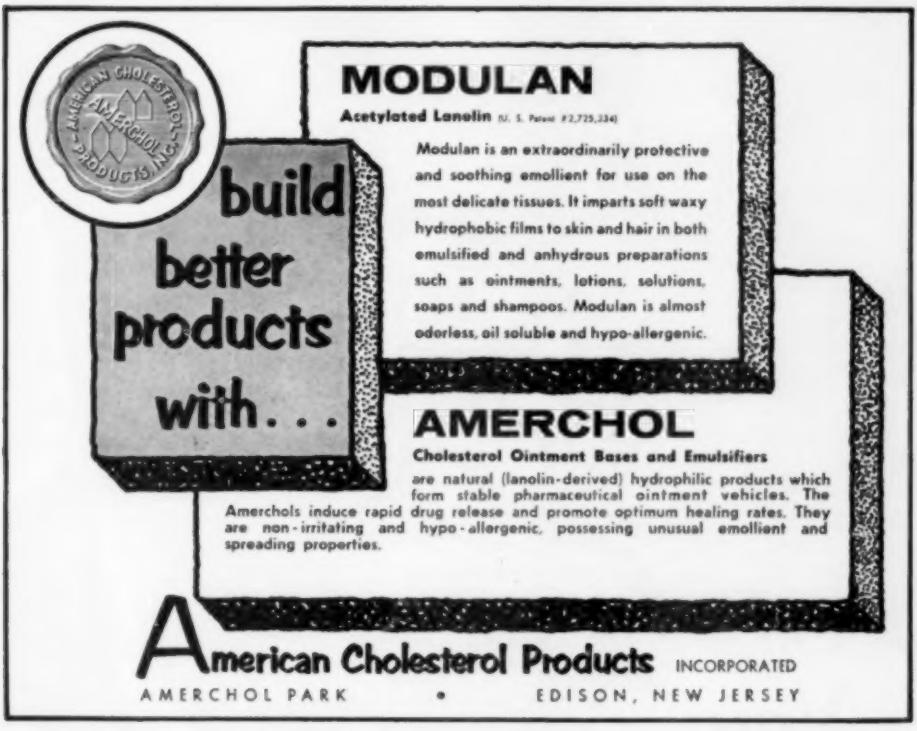
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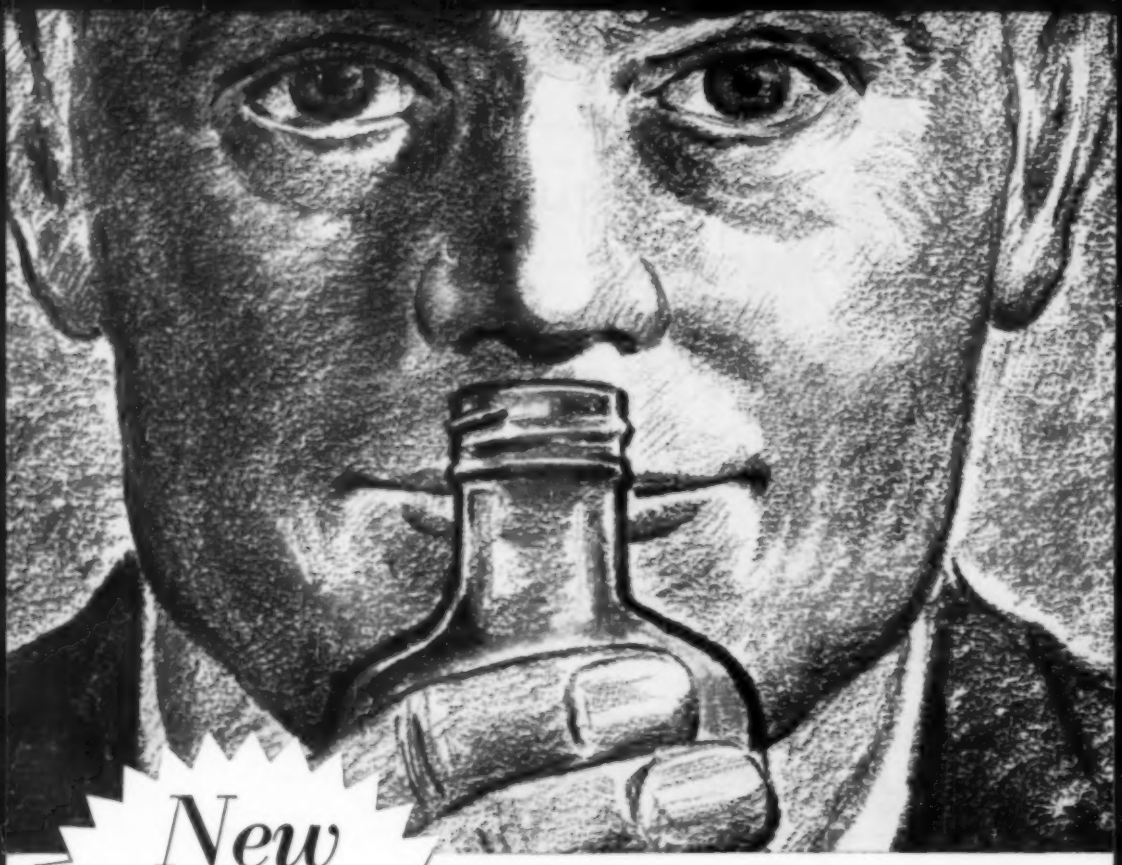

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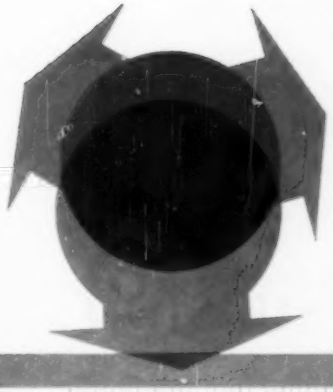

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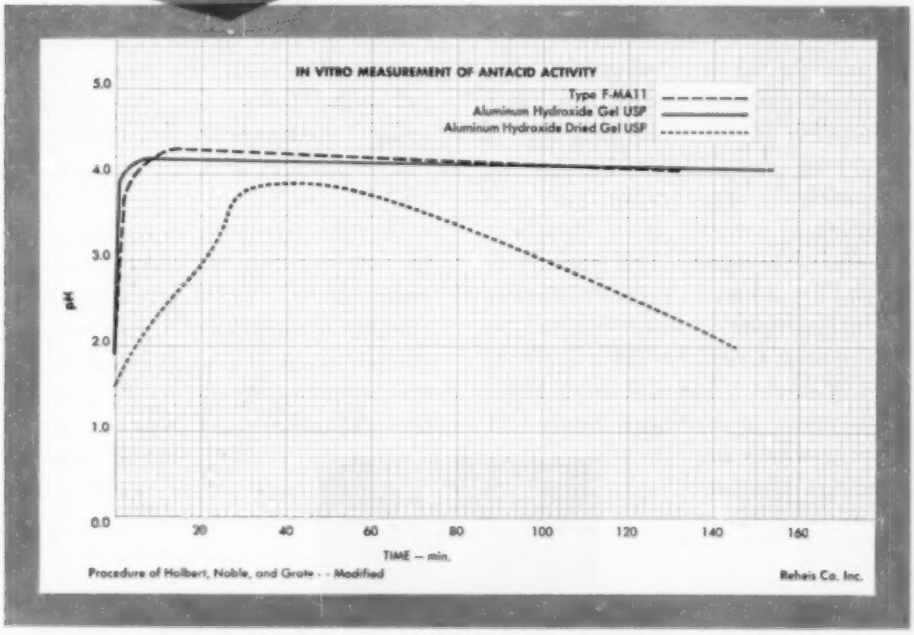

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JOURNAL OF **Pharmaceutical Sciences**

February 1961 volume 50, number 2

Review Article

Role of Metal-Binding in the Biological Activities of Drugs

By WILLIAM O. FOYE

"That metal complexes can be chemically more vigorous than the metal cations on their own is evident from consideration of the metal-containing enzymes" (3).

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Received from the Department of Chemistry, Massachusetts College of Pharmacy, Boston.

INTRODUCTION

Since the discovery that metal ions are active constituents of many enzymes the use of metal chelation[1] or binding mechanisms to explain the biological and pharmacological effects of molecules capable of metal ion chelation has grown in importance. The necessity for small amounts of specific metals for the operation of many metabolic reactions has also given rise to postulations involving chelation chemistry. It is the purpose of this review to consider the chelation mechanisms that have been offered to explain, in part at least, the effects of a number of biologically active molecules, particularly those which have been used as medicinals.

Among the metal ions which have been found to exert these catalytic or otherwise "activating" effects are a number of the transition metals, such as Fe, Co, Zn, Cu, Mn, and Mo, which are also the elements which form coordination, or chelate, compounds with the greatest facility and are also the essential "trace metals" for the human. Magnesium, also capable of forming coordination compounds, is also believed to act via chelation mechanisms and Al and Cr have also been implicated in this type of reaction. Much of the evidence regarding their activities, furthermore, indicates that these metal ions are effective in a bound form rather than as free ions (5–10). Also, in this regard, experimental evidence exists to show that metal chelates of riboflavin (11) and pyridoxal (12) catalyze the same biologic reactions as their respective enzymes.

Possible modes of action in which metal ion chelation may be involved include: (*a*) transient combination of a chelating molecule (ligand) with the metal of an enzyme without removal of the metal from the enzyme; (*b*) combination of a chelating molecule with metal ions in the cellular environment and removal of the metal ions which thus deprives the cell or organism of the metal; (*c*) combination of a chelating molecule with metal ions to give a complex of suitable polarity to transport either a metal ion or the molecule to or through a cell boundary; (*d*) stabilization of either a chelating molecule or a valence state of a metal ion through chelation; (*e*) combination of a chelating molecule and metal ions to give an "unsaturated" chelate (generally a 1:1 ratio of ligand to metal in the case of a divalent metal, and 2:1 ratio for a trivalent metal) which exerts a toxic or catalytic effect *per se*; (*f*) combination of a chelator and metal ion to alter the oxidation potential of the metal.

Other possible pathways in which biological reactions involve metal chelation undoubtedly exist and should become known as evidence for them is uncovered. The use of chelating agents for the removal of toxic metal ions from the body has been recently covered in the reports of several conferences (13–15) and will not be treated here. Reviews concerned with chelation in pharmacological action (4) and antimicrobial action (16) have appeared in the past five years as well as a discussion of the modification of toxic as well as medicinal effects by metal chelation (3). Chelation effects in plant cells have also been discussed (14, 16). In view of this wealth of coverage this discussion will be concerned with presenting certain views regarding chelation as a mechanism of drug action and considering in detail only those cases where sufficient evidence has been presented. A complete review of the literature on chelation effects in biological activities has now grown well beyond the limits generally imposed on a review paper.

INDICATIONS OF CHELATION MECHANISMS

Some general indications that metal ion chelation or complexation may be involved somewhere in the course of a medicinal substance through a living organism include the following. The determination of stability constants for the reactions of organic chelators and metal ions [made possible by the work of Bjerrum (17)] that exceed those of naturally-occurring cellular chelators for the same metals provides good indication that metal chelation is important. Table I summarizes the constants already determined for some medicinal substances with biologically-important metal ions and they may be compared with the constants found for representative cellular chelators with these metals in Table II. Constants found for the reactions of a number of cellular chelators with metals have been determined by Albert (18–20).

The finding of oil-water partition coefficients of metal chelates favorable to their transportation to, through, or within the cells also points to the possibility of a chelation mechanism. This has been utilized in only a few cases, however, and this method of approach awaits what should amount to some very revealing investigations. In this connection it should be mentioned that the determination of coefficients of a series of chelating agents not combined with metal ions can be

[1] The chemistry of chelation has been adequately described in recent texts by Martell and Calvin (1) and by Bailar (2). Excellent summaries of this topic have been written by Albert (3) and Chenoweth (4).

TABLE I.—STABILITY CONSTANTS OF SUBSTANCES OF THERAPEUTIC VALUE WITH HEAVY METAL CATIONS

Ligand	log K_1 (Water, 20–30°) Cu^{++}	Zn^{++}	Co^{++}	Fe^{++}	Mn^{++}	Fe^{+++}	Ref.
8-Hydroxyquinoline	12.2	..	9.1	8.0	6.8	12.3	(20)
Salicylic acid	10.6	6.9	6.8	6.6	5.9	16.4	(123)
Gentisic acid	7.0	..	..	..	..	10.8	(124)
p-Aminosalicylic acid	..	..	..	..	..	16.1	[a]
o-Thymotic acid	9.7	..	..	..	..	15.8	[a]
Methyl salicylate	5.9	..	..	..	..	9.7[b]	(123)
Isonicotinyl hydrazide	8.0	5.4	4.8	..	..	...	(69)
Tetracycline	7.8	4.9	5.4	5.3	4.4	9.9	(45)
Chlortetracycline	7.6	4.5	4.8	5.7	4.3	9.4	[c]
Oxytetracycline	7.2	4.6	5.1	5.6	4.3	9.6	[c]
Dimethyldithiocarbamic acid	11.4	...	...	..	..	...	(146)
2-Mercaptoethylamine	..	10.2	7.7	..	..	...	[d]

[a] Foye, W. O., and Turcotte, J. G., unpublished. [b] Ågren, A., *Acta Chem. Scand.*, **9**, 39(1955). [c] Albert, A., *Nature*, **172**, 201(1953). [d] Gonick, E., Ph.D. thesis, Pennsylvania State College, 1951.

TABLE II.—STABILITY CONSTANTS OF REPRESENTATIVE CELLULAR CHELATORS WITH METAL CATIONS

Ligand	log K_1 (Water, 20–30°)[a] Cu^{++}	Zn^{++}	Co^{++}	Fe^{++}	Mn^{++}	Fe^{+++}
Glycine	8.5	5.2	5.1	4.3	3.2	10.0[b]
Cysteine	..	9.8	9.3	6.2	4.1	..
Histidine	10[c]	6.7	7[c]	5[c]	<4	4.7[b]
Glutamic acid	..	5.4	5.1	4.6	3.3	12.1[b]
Histamine	9.6	5.7	5.3	..	..	..
Glycylglycine	6.3[c]	3.7	3.0	..	2.1	..
Pteroylglutamic acid	4.4[c]	4.2[c]	4.6[c]	4.5[c]	3.5[c]	..
Riboflavin	7[c]	5.6	3.9	7.1	3.4	..
Hypoxanthine	6.2	..	3.8	3.9	2.4	..
Guanosine	~6	4.6	3.2	4.3	..	..
Lactic acid	1.8[c]	2.0	0.9[c]	..	..	6.4
Oxalic acid	6	4.9	4.5	4.5	3.9	9.4

[a] Values for log K_1 were considered to give a better comparison than log K_2 (overall) values, which are not always available. These were taken mainly from Bjerrum, J., Schwarzenbach, G., and Sillén, L. G., "Stability Constants," Part I: Organic Ligands, The Chemical Society, London, 1957. [b] Perrin, D. D., *J. Chem. Soc.*, **1958**, 3125. [c] Estimated from log K_2 values.

valuable and may point to more effective molecules, but extrapolation to the relative coefficients of the chelates themselves is not always valid.

The observation of metal ion activations or reversals in biological reactions is generally a good indication that chelation of the metal is taking place. These observations have become so common however, that further mention of them should not be necessary here.

The administration of some chelating substances complexed with metal ions has been found effective and provides very good indication of the importance of metal chelation. Notable examples of this practice are the combination of some fungicides with metals, such as 8-hydroxyquinoline and sodium dimethyldithiocarbamate, the use of a streptomycin-copper complex as a growth factor, and the use of zinc-bacitracin as an antibacterial. Where administration of metal ions themselves is the object chelates have frequently been used. Introduction of Fe, Ca, Cu, Pb, Sb, and Bi into the human has often been done via their chelates (21, 22) with such ligands as citric, lactic, tartaric, gluconic, and thioglycollic acids, and 1,2-dihydroxybenzenes.

The study of structure-activity relationships can also be of value in pointing to a chelation mechanism. Where modifications in electronic or steric effects are made in a chelating molecule an increase in chelating ability accompanied by an increase in biologic activity should offer good indication that metal chelation *in vivo* cannot be overlooked. Although few examples of this approach are known it is anticipated that more such studies will be carried out.

ANTIBACTERIAL COMPOUNDS

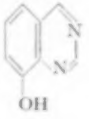
8-Hydroxyquinoline.—The mutual effects of antimicrobial compounds and metallic cations and the possible metal chelation mechanisms involved have been well described by Weinberg (16). Since that review appeared (1957), 8-hydroxyquinoline (oxine) has remained the best defined, and documented, case where metal chelation is essential for antibacterial and antifungal effects. In his now classical experiments

Albert first showed that the six isomers of oxine, which are incapable of chelation, had no antibacterial action (23). The ability of oxine to chelate metals was then blocked by methylation of both the hydroxyl group and the nitrogen atom and, in each case, antibacterial activity was lost. The necessity for chelation in the antibacterial action of oxine appeared to be established.

After having determined the stability constants with metal ions of a large number of potential cellular chelators, mainly amino acids and purines (18–20), Albert was able to show that the constants for the reaction of oxine with the trace metals were higher (20). This indicated the ability of oxine chelates to exist under biological conditions although chelation, either *in vitro* or *in vivo*, is controlled as much by the ionization constant of the chelator as by the stability constants of the chelates. [It is necessary for the ligand to be ionized before chelation can take place (18)].

The possibility that the activity of oxine is due to its ability to remove essential metals from the microorganisms, suggested by Zentmyer (24), or to a toxic effect of the metal chelate was also explored. In both the bacteriostatic (25) and bactericidal (26) actions of oxine the latter possibility appeared to be operative. Experiments conducted in metal-depleted media against staphylococci and streptococci revealed that neither oxine (I) nor iron ions alone were harmful to the organisms but essentially equimolar concentrations of the two were lethal at high dilution ($M/100{,}000$). Variation of the ratios of ligand and metal removed the toxic effects to the cells when either ligand or metal was in large excess. This was interpreted by Albert to mean that the unsaturated 1:1 (II) or 2:1 (III) chelates were toxic whereas the 3:1 (IV) chelate, also chemically unreactive, was nontoxic. When sufficient iron was added to shift the equilibrium in favor of the 1:1 chelate bactericidal action was restored.

For Gram-positive bacteria, ferrous, ferric, and cupric ions gave toxic combinations with oxine, and trace amounts of cobaltous ion suppressed the activity. Gram-negative bacteria are much less susceptible to oxine but a metal requirement appears to exist since the toxicity of oxine is suppressed by Fe^{++}, Zn^{++}, or Cu^{++} ions. Others have also reported the necessity for iron or cupric ions for the antibacterial action of oxine (27, 28). Toxicity to fungi and yeasts by oxine apparently requires only cupric ions (29, 30).



V

Investigations that might lead to knowledge of the site of action of iron-oxine were conducted by modification of the liposolubility of oxine. Introduction of a highly hydrophilic group, the 5-sulfonic acid, removed antibacterial properties altogether, although the metal-binding ability remained equal to that of oxine (20). A series of oxine derivatives in which the oil/water partition coefficients were varied by introduction of additional hetero nitrogen atoms was then prepared. These aza-oxines (such as V) were found to have antibacterial effects which decreased as the liposolubility decreased (31) (see Table III). Introduction of short alkyl groups was effective in restoring both liposolubility and antibacterial properties and the two effects ran parallel as long

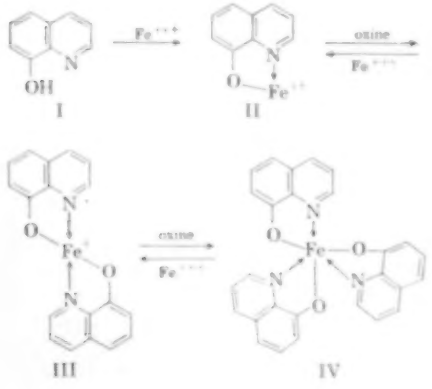


TABLE III.—CORRELATION OF BACTERIOSTATIC ACTION WITH LIPOSOLUBILITY OF AZA-OXINES[a]

Compound	Partition Coefficient, Oleyl Alcohol/Water	Lowest Inhibitory Dilution, 1/M[b]	log K_1 (Ni^{++})
Oxine	67	200,000	9.8
5-Aza-oxine	<0.02	<800	5.8
7-Aza-oxine	0.1	<800	6.7
6-Aza-oxine	1	<800	5.9
3-Aza-oxine	5	13,000	7.6
2-Aza-oxine	6	13,000	7.8
4-Aza-oxine	8	6,400	7.6
4-Methyl-2-aza-oxine	16	25,000	8.1
4-Methyl-3-aza-oxine	17	50,000	7.9
4-Propyl-3-aza-oxine	135	100,000	7.9
7-Allyl-3-aza-oxine	310	100,000	7.9

[a] From Albert, A. (3). [b] Against *Streptococcus pyogenes* in broth, pH 7.3.

as the chelate stability constants remained sufficiently high.

As a result of these observations Albert suggested that the action of oxine was intracellular, the less polar 3:1 iron or 2:1 copper chelates assisting in the penetration of the cell wall, and then shifting to the more toxic 1:1 or 2:1 chelate within the cellular environment. Presumably, an excess of oxine would keep the chelate at the 3:1 ratio (in the case of iron), which is nontoxic, and an excess of iron would shift the equilibrium to the 1:1 chelate, which is probably toxic but would be too polar to penetrate the cell wall (32).

Other evidence exists that oxine acts at the cell wall or cytoplasmic membrane. Beckett (33) has shown that the 1:1 oxine-iron chelate reacts with the *Staphyloccocus aureus* cell and liberates oxine rather than iron. This is inconsistent with the idea that the 1:3 iron-oxine chelate forms and penetrates the cell since free iron would then be liberated from the 1:1 chelate. The fact that iron was necessary for the uptake of oxine by the cell suggested that the iron-oxine complex became bound to an enzyme, possibly a flavoprotein, thus preventing reaction with the normal substrate. Greathouse, *et al.* (34), have also shown radioactive oxine to be taken up by the *Aspergillus niger* cell.

As to the role the oxine chelate plays after reaching or entering a cell wall or interior, we have only very indirect or indicative evidence. Bernheim and Bernheim (35), for instance, found that iron and oxine together catalyze the oxidation of −SH groups in nucleoproteins, whereas iron alone is ineffective. In this connection it has been noted that traces of cobalt can inhibit iron- or copper-catalyzed oxidations of mercapto compounds (36), which offers a possible explanation for the as yet unexplained antagonism between Co^{++} and either iron-oxine or copper-oxine. Vajda and Nógrádi (37) have suggested that copper-oxine replaces Co^{++} in the prosthetic group of an enzyme.

A possibility that has been largely overlooked, however, is suggested by the fact that substitutions in the 2-position of oxine do not hinder chelation appreciably or lower liposolubility but do depress antibacterial activity considerably (23). This would indicate that further combination of the chelate takes place with an essential group, possibly of an enzyme. Beckett (38, 39) has already provided evidence that oxine is bound by the "erythrochelatin" of red cells, which accounts for its inactivation in the blood (25) and suggested binding of oxine to flavoprotein enzymes (33).

Other Antibacterials.—The effects of metal ions in the bacterial growth inhibitions caused by the tetracycline antibiotics was amply discussed by Weinberg (16) in 1957. Chelation mechanisms have been proposed for the action of the tetracyclines on bacterial growth and enzyme systems by a number of investigators (40–44) but convincing proof of their reality has not been found. It should be considered, however, that the stability constants of the tetracyclines with divalent and trivalent metals (45) are sufficiently great for the tetracyclines to compete for metal ions with the amino acids and proteins of the cells.

In the various enzyme systems inhibited by the tetracyclines the inhibitions may be reversed by Fe^{++}, Mg^{++}, Mn^{++}, and Ca^{++} ions (16), which points to possible chelations of these metals or metalloenzymes by the tetracyclines. The tetracyclines, however, have been found active in iron-depleted media (45) against *Staphylococcus aureus*. This result is not necessarily inconsistent with the previous reversals of inhibition shown by Fe^{++} ion, since it is extremely difficult to remove all the metal ions from aqueous media (3), and antibiotic preparations always carry a certain amount of trace metals with them (46). In the case of tetracycline this amounts to 10 p. p. m., which should be sufficient for metal chelate effects and certainly for catalyzing oxidation-reduction reactions. Saz and Slie (47), for instance, have shown a nitro reductase to be inhibited by the tetracyclines and have postulated that the inhibition takes place through the binding of Mn^{++} ion by the tetracycline since Mn^{++} stimulated the reducing ability of the enzyme.

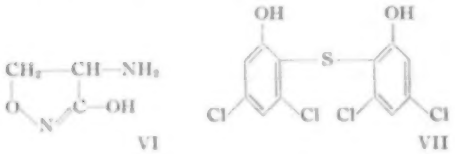
Other antibiotics and antibacterials where metal ion activations and reversals have been noted and where chelation of metals is a possibility include: penicillin (48–50), bacitracin (48), polymyxin (51), novobiocin (52, 53), 1-nitroso-2-naphthol (54), kojic acid (55), actidione (55), juglone (55), picolinic acid (55), cycloserine (55), morin (55), patulin (55), neomycin (56), and aspergillic acid (57). Cycloserine (VI) is known to chelate Cu^{++}, Zn^{++}, Co^{++}, Cd^{++}, and Ni^{++}, and possibly Fe^{++}, Fe^{+++}, Mn^{++}, and Mg^{++} ions (55–59) as well. The preformed copper-cycloserine chelate has no antibacterial properties, however.[2]

The capacity of penicillin for complex formation with cupric ion has been measured by Weiss, *et al.* (60), by means of an ion exchange method. Comparison of the stability constants

[2] W. E. Lange, personal communication.

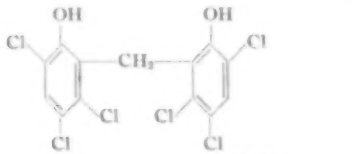
with those for related heterocyclic carboxylic acids and cupric ion indicate that a ring-opened product, the corresponding penicilloic acid, was responsible for the chelation of Cu^{+}. Cleavage of penicillins to penicilloic acids, which are not antibacterial, is known to be caused by cupric ion (61). Although the action of penicillin has been traced to an acylation reaction resulting in the lysis of bacterial cells metal chelation as one essential step in the process has not been excluded.

The chlorinated bisphenol antibacterials also appear to act via a chelation mechanism. 2,2′-Thiobis(4,6-dichlorophenol) (VII) was found to chelate Fe^{++}, Fe^{+++}, Cu^{++}, Mn^{++}, and Co^{++} ions and hexachlorophene (VIII) chelated Fe^{++}, Fe^{+++}, and Cu^{++} ions (62).



VI

VII



VIII

The thiobisphenol was bactericidal to *Staphylococcus aureus* in distilled water and metal-depleted broth at 1 p. p. m. and it was stated that it was not dependent upon the presence of trace metals in the medium for activity, in contrast to oxine (63). Both the copper and iron chelates showed the same activity as the thiobisphenol and, unless metal chelation took place (probably within the bacterial cell), the activity of the chelates would be expected to be less. Both phenols were suppressed in activity by Fe^{++} alone among the metals tried, which pointed to the inhibition of iron-containing enzymes by these substances. The retention of activity of these phenols in mildly alkaline, or soap, solutions was also attributed to iron chelation.

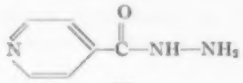
Evidence has been presented by Counter, *et al.* (64), that a metal chelate of a nonbacteriostatic agent can exert a greater growth-inhibitory effect than a molecular equivalent of the metal ion. This was demonstrated by the action of a cobalt complex of methionine and a copper complex of biotin against *E. coli* and *S. aureus*. A comparable experiment with a copper complex of glycylalanine showed the inhibitory effect of the complex to follow that exerted by the free metal ion alone over a period of eight hours. This pointed to the fact that metal chelate structures, *per se*, are capable of toxic effects on cells. The antibacterial action was postulated as being due to an increase in liposolubility of the metal ion by chelation which allowed it to enter the cellular environment and be liberated in larger quantities than the cell can tolerate.

Further indication of this effect was provided by the lack of antibacterial properties of a *p*-aminosalicylic acid copper complex (XIII) of low fat solubility and the possession of antibacterial properties of a *p*-aminosalicylic acid copper chelate (XIV) of much greater fat solubility. Presumably, the polar complex was not capable of penetrating the cell whereas the chelate of lower polarity could invade the cell and liberate metal ions since its inhibitory pattern followed that of the metal ion.

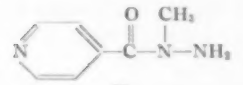
ANTITUBERCULAR COMPOUNDS

Since the postulation of Carl and Marquardt (65) of a direct relation between the activities of most antitubercular compounds and their ability to form copper complexes practically all the compounds which have been used against tuberculosis have also been shown to be influenced by metal ions (16). Clear-cut demonstrations of metal chelation mechanisms of action, however, are difficult to find but much indicative evidence of their involvement in antituberculotic action is at hand.

Isonicotinic Acid Hydrazide.—Isonicotinic acid hydrazide (isoniazid, INH) (IX) appears by the usual criteria to function as a chelating agent. Growth inhibition of *Mycobacterium tuberculosis* by isoniazid, for instance, is suppressed by the presence of Fe^{+++}, Mn^{++}, Cu^{++}, and Co^{++} ions *in vitro* (66, 67). Stimulatory effects by metal ions have been noted in the ability of INH to inhibit respiration of *M. tuberculosis* (67) and to inhibit the action of mammalian or microbial catalase (68). Albert has determined stability constants for INH and a number of the trace metals and found them only slightly less than those determined for the common amino acids (69, 70). Cymerman-Craig, *et al.*, added further support to a chelation hypothesis by showing that INH derivatives incapable of metal chelation (such as 1-isonicotinoyl-1-methylhydrazine) (X) were also devoid of antitubercular activity *in vitro* (71). Rubbo, *et al.*, also showed that the preformed copper chelate of INH was as effective in tuberculotic mice as INH itself,



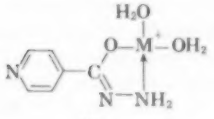
IX



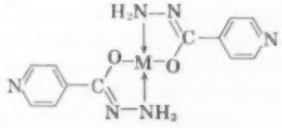
X

using a healing-ulcer (72) as well as a more conventional technique (73).

Other studies carried out *in vivo* with preformed metal chelates gave results of a less conclusive nature. INH chelates of Cu^{++}, Co^{++}, Fe^{++}, and Zn^{++}, having both 1:1 (XI) and 2:1 (XII) ligand-metal ratios, all showed approximately the same antitubercular activity when fed to mice as INH itself (74). Such lack of specificity would be surprising if INH acted against mycobacteria as a toxic metal chelate.



XI



XII

The toxicity patterns of the chelates in mice in general followed those found for the corresponding metal ions although the toxic effects of the chelates did not appear as rapidly as those from the metal ions and, in fact, required a day or more to become evident. These facts suggested that the INH chelates liberate some, if not all, of their metal ions *in vivo* but apparently not rapidly enough to exclude the chelate as an active entity in the course of antitubercular action. Equilibrium studies showed the tendency of the 2:1 copper chelate to shift to the 1:1 chelate in aqueous media over a period of time, which led to the postulation that a 1:1 metal chelate was an active species. For transport through a cell wall, however, the 2:1 chelate would be required, as shown by the much higher oil/water partition coefficients in comparison to the 1:1 compounds.

Further evidence of the liberation of metal ions *in vivo* by an INH chelate was obtained using the 2:1 cobalt chelate in broth cultures of *E. coli* and *S. aureus* (64). That the liberation took place within the cells was indicated by a parallel study in which a polar copper complex of *p*-aminosalicylic acid (XIII), incapable of penetrating the cells, had no antibacterial activity whereas the fat-soluble copper chelate (XIV) was active.

Although the necessity for chelating ability appears to have been established for INH the exertion of its antitubercular effects through a toxic metal chelate has not. The possibilities remain that chelation is involved in transport of INH into the cell or that INH acts as a transient chelator inhibiting pyridoxal, which it resembles structurally. Transamination and other reactions catalyzed by pyridoxal have been postulated (12) as occurring through a chelation mechanism, and the antitubercular action of INH is inhibited by pyridoxal, as well as by α-ketoglutarate and pyruvate (75). Depressions of the glutamic-oxalacetic transaminase levels of humans (76) and amino acid metabolism of rats (77) have also been reported for INH. Hicks and Cymerman-Craig (78) however observed 50 per cent inhibitions of transaminase activity *in vitro* by a series of hydrazides, including INH and its isomers, and concluded that there is no correlation between antituberculous and antivitamin B_6 activity among these hydrazides, unless the bacterial cell is selectively permeable to INH.

The fact that the isomers of INH have as good or higher avidities for metals but much less antitubercular activity (70) suggests that only transient chelation, requiring a relatively low stability constant, is required. Other possibilities for the mode of action of isoniazid have been mentioned. Maher (68) for instance, suggested that isoniazid forms a metal chelate which competes with peroxide for sites on catalase molecules. This would result in accumulation of peroxide and death of the bacilli. Vischer (79), however, believes that isoniazid resistance or susceptibility in mycobacteria is more closely related to peroxidase than to catalase, and demonstrated the presence of peroxidase in normal mycobacteria but its absence in all mycobacteria resistant to INH. Coleman (80) has proposed two ways in which isoniazid might act: (*a*) by interference with metal pyridoxal-catalyzed transaminations, and (*b*) by interference with cytochrome-catalyzed respiration through chelation of Fe^{+++} ion.

An increase in the antitubercular activity of INH by the presence of agents which formed complexes with copper was noted by Leonardi (81). This suggests that metal chelation might be interfering with the action of INH against the mycobacteria but such an hypothesis is difficult to reconcile with the fact that blocking of chelating

ability removes the antitubercular activity of INH (71). Furthermore, if metal chelation is involved in transport of INH, agents aiding in the liberation of INH from the metal within the cellular environment would tend to promote its activity.

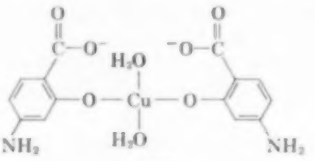
p-Aminosalicylic Acid.—Activations by Cu^{++} (82) and Co^{++} (83) ions have both been shown for the tuberculostatic effect of *p*-aminosalicylic acid (PASA) *in vitro*, but the majority of ions suppress it (84). Roth, *et al.* (82), postulated a chelate structure for the reaction product of PASA and Cu^{++} in neutral or alkaline media, but it was later found by Foye (85) that the structure was more likely an open-chain complex (XIII). Chelate or cyclic structures were obtained from PASA and metal ions in acid media (86) (XIV), however, and antitubercular activities in mice of both a number of metal complexes and chelates were compared. The Cu^{++} chelate was found to have antitubercular activity equal to or greater than that of PASA itself, whereas the Fe^{++} chelate showed lesser activity and the metal complexes were not only inactive but were highly toxic to mice. The difference in activity was explained as due to the greater liposolubility of the chelates (see Table IV), the complexes being too polar to penetrate the cells.

Table IV.—Correlation of Antitubercular Action with Liposolubility of Metal Chelates

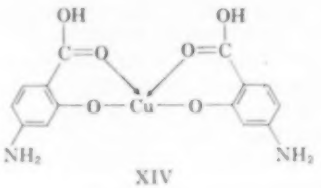
Compound	Partition Coeff. $\times 10^3$, Oleyl Alc./ Water	Mortality in Mice, %*	Mean Survival Time, Days
$(PAS)_2Cu$ complex	8	60	15.6
$(PAS)_2Fe$ complex	..	100	13.7
Controls	..	90	14.3
$(PAS)_2Cu$ chelate	244	0	23+
$(PAS)_2Fe$ chelate	174	60	20.6+
PASA	..	0	21+
Controls	..	80	20.3+

* The compounds were fed to mice infected with *M. tuberculosis* H37Rv at levels of 0.1–0.5% in the diet.

It is also interesting that the Cu^{++} chelate exhibited both a greater antitubercular activity and higher liposolubility than the Fe^{++} chelate. This led to the postulation that the 1:1 Cu chelate was an active species since it had previously been shown that excess Cu^{++} ion increased the tuberculostatic activity of PASA tenfold (82) and excess Cu^{++} ion would shift the chelation equilibrium in favor of the 1:1 compound. In view of the finding that *p*-aminosalicylic acid replaces *p*-aminobenzoic acid in some species of bacteria (87) a chelate mechanism would more likely be involved in transport of the PASA and the 2:1 chelate would then be the important species.



XIII



XIV

Recent determinations in our laboratory (unpublished) of stability constants of metal ions with eight nuclear-substituted salicylic acids have shown that the 4-amino derivative has the greatest avidity for Fe^{+++} ions of any of the salicylates used. The constant for the Fe^{+++} chelate (see Table I) was much greater than that of the common amino acids with Fe^{+++} ion (the corresponding constant with Cu^{++} ion was not obtained because of the insolubility of the Cu^{++} chelate).

Tests against other bacteria (ten common organisms were used) revealed that both the Cu^{++} complex and chelate had antibacterial properties on the agar plate, but in broth culture only the chelate showed appreciable activity (64). This again was interpreted as being due to the greater fat solubility of the chelate.

Other Antituberculotics.—Some evidence of chelating possibilities for streptomycin has been mentioned by Weinberg (16) and little can be added at this time. Small enhancement of activity by Co^{++} has been observed in the action of streptomycin against *Micrococcus pyogenes* (48) and *Vibrio fetus* (88), but it should be borne in mind that traces of cobalt can inhibit oxidations of cell constituents, such as thiols and ascorbic acid (36), and minor activations by cobalt could well indicate lesser destruction of the molecule through oxidation. The suppression of catalase and dehydrogenase activity in *E. coli* and *Shigella* organisms by streptomycin (89) may be indicative of interference by complexation with the metal ions involved.

Streptomycin complexes with Cu^{++}, Co^{++}, and Ni^{++} were isolated and found to have a less

potent antitubercular effect but more prolonged blood levels in guinea pigs then streptomycin sulfate (90). This was probably due to a slow liberation of the streptomycin from the complex and cannot be construed as evidence of a chelation mechanism for the antitubercular effects of this compound.

A number of thiosemicarbazones having some antitubercular properties have also been shown to be complexing agents for copper (91, 92). *p*-Acetylaminobenzalthiosemicarbazone,[3] in particular, was shown to be more active against mycobacteria *in vitro* as the copper complex (92). Marquardt (93), however, attributed the anemia observed in humans after oral treatment with *p*-acetylaminobenzalthiosemicarbazone to be due to complexing of copper in the body.

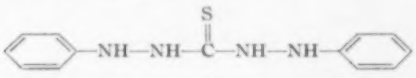
Erlenmeyer, *et al.* (94, 95), have prepared a large number of compounds capable of metal chelation, based largely on the structure of 8-hydroxyquinoline, and found their tuberculostatic activities *in vitro* to be enhanced four to eight hundred-fold in the presence of Cu^{++} or Co^{++} ions. Cymerman-Craig (96) noted that among an extensive series of aromatic amines showing tuberculostatic properties that the most active compounds were chelating agents. It has also been observed by Garattini and Leonardi (97) that most compounds capable of chelating heavy metals exert an inhibitory effect on *M. tuberculosis* and *M. paratuberculosis*.

A series of metal chelates of some *o*-hydroxyphenylazo naphthols and phenanthrols, inactive in the unmetallized state, have shown antitubercular effects *in vitro* (98). Copper chelates of phenylazo cresols and resorcinols have also been reported to be tuberculostatic (99). Another example where the copper chelate showed a much greater antitubercular effect in mice than the unmetallized compound was found with *p*-aminophenyl-*p*-hydrazidomethylaminophenyl sulfone (100).

DIABETOGENIC AND ANTIDIABETIC AGENTS

Diabetogenic Agents.—A number of molecules having strong abilities to chelate metal ions have been found to produce in animals diabetogenic effects resembling those effects produced by alloxan. Diphenylthiocarbazone (dithizone) (XV), for instance, has been reported to damage the beta cells of the pancreatic islets and bring about diabetes mellitus (101–103). Since dithizone is a powerful chelating agent for zinc it is not unexpected that serum zinc levels in dogs increased after dithizone injection and the loss of zinc from the islet cells paralleled the dithizone dose (103).

8-Hydroxyquinoline, another powerful chelator, has also been found effective in producing diabetes mellitus (102, 104). Matsuo, *et al.* (105), have shown, furthermore, that oxine derivatives whose ability to chelate had been blocked (e. g., 8-methoxyquinoline) lost their diabetogenic effects. Other studies by Root and Chen (102), Kadota and Abe (106), and Iwamoto and Adams (107) have also shown metal chelating ability to parallel diabetogenic activity.



XV

Alloxan (XVI) itself undergoes chelation with heavy metal ions such as Co^{++}, Ni^{++}, Cu^{++}, and Zn^{++} (108). Chelates of alloxan having a 1:1 ratio of alloxan to metal have been isolated. Administration of the Co^{++} and Ni^{++} chelates to rabbits in nondiabetogenic doses produced increases in the blood sugar level equivalent to, or greater than, that from alloxan but the periods of action were much longer (108) (see Table V). Greater increases in blood sugar levels were obtained from administrations of cobalt chelates of related structures containing the alloxan ring, namely riboflavin and the dichloro analog of riboflavin (XVII). It is interesting that the cobalt chelate of alloxanic acid, which is formed from alloxan in alkaline media, showed no increase in blood sugar levels. These observations led to the postulation that alloxan

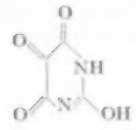
TABLE V.—COMPARISON OF DIABETOGENIC EFFECTS OF METAL CHELATES AND CHELATORS

Compound	Time, hr.	Increase in Blood Sugar Level, mg. %[a] 1	2	3	4
Alloxan		48	4	..	..
Alloxan-Co		..	..	35	..
Alloxan-Ni		64	42	..	..
Alloxanic acid-Co		..	0	..	..
Riboflavin-Co		72	..	..	−26
Dichloro analog[b]		30	..	..	−36
Dichloro analog-Co		..	..	224	..
Hydroxyethyl analog[c]		..	0	..	..
Hydroxyethyl analog-Cu		85	80	37	..
Aniline analog[d]		..	0	..	..
Aniline analog-Cu		75	100	30	..

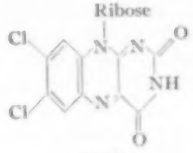
[a] The compounds were administered i. p. to rabbits with the exception of the second and seventh compounds which were given i. v., at a dose of 20 mg./Kg.
[b] 6,7-Dichloro-9-(1-D-sorbityl)-isoalloxazine.
[c] 6,7-Dichloro-9-(2′-hydroxyethyl)-isoalloxazine.
[d] 2-Amino-4,5-dichloro-N-(2′-hydroxyethyl)-aniline.

[3] Marketed as Tibione by Schenley.

exerts its diabetogenic effects in the form of a 1:1 metal chelate, possibly combining with an essential cellular group, such as thiol, through the intermediary binding capacity of the metal ion. In this connection, combinations of alloxan with cysteine or glutathione (109) or with 2,3-dimercaptopropanol (110) are nondiabetogenic. Zinc, however, protects rabbits from the initial hyperglycemia caused by alloxan but does not affect the final production of diabetes (111).



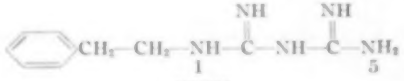
XVI



XVII

Other structures related to riboflavin showed similar blood sugar elevations, as does alloxan in rabbits, when administered as the 1:1 copper chelate and, like riboflavin, were inactive in this regard when not previously chelated (112). The antitubercular *p*-acetylaminobenzalthiosemicarbazone, also known to be a metal complexing agent, has caused diabetes mellitus in tuberculous patients (113) and two powerful chelators, 6-hydroxy-1,7-phenanthroline and 1-hydroxyacridine, were reported to cause hyperglycemia and destructive changes of the pancreatic islets in rabbits (114). Other chelating agents had no effect on blood sugar.

Antidiabetic Agents.—The biguanides such as 1-*p*-chlorophenyl-4-isopropylbiguanide,[4] amylbiguanide, and more recently *β*-phenethylbiguanide[5] (XVIII) have shown significant anti-



XVIII

diabetic effects, the latter compound having been tested the most extensively as an oral hypoglycemic agent. The unsubstituted biguanide structure has been found to chelate a number of heavy metals (115) but *β*-phenethylbiguanide gives evidence of forming chelates of appreciable stability only with copper and nickel ions (116, 117). It is unfortunate that stability constants are not available for these chelates but the copper chelate of *β*-phenethylbiguanide was found, by a comparative method, to exceed slightly the stability of copper-glycine at acid pH (117).

[4] Marketed as Paludrine by Ayerst.
[5] Marketed as DBI by U. S. Vitamin and Pharm.

A series of substituted biguanides, having from one to four alkyl substituents located on the N_1 and N_5 atoms, has been synthesized (118) and tested for their ability to chelate Cu^{++} ion (117). In a series of twelve such compounds chelating ability paralleled hypoglycemic activity with two exceptions. No definite conclusion regarding the importance of metal ion chelation in the hypoglycemic action of the biguanides was drawn but the relative stability of the copper-*β*-phenethylbiguanide chelate, in comparison to copper-glycine, indicates that it is capable of existence under biological conditions.

Weinberg, *et al.* (116), have carried out antibacterial studies with *β*-phenethylbiguanide in which they found no effect on activity against several common organisms by copper or nickel but the antimicrobial effects were depressed by iron. Inhibitions of iron-containing enzymes by *β*-phenethylbiguanide have also been reported (119, 120). Weinberg, *et al.*, pointed out that the chelating ability of *β*-phenethylbiguanide might be considered either beneficial or detrimental to the hypoglycemic effect.

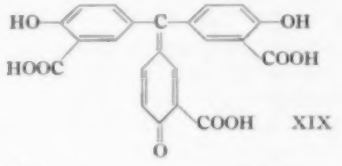
Other metal-binding agents, including the salicylates, are known to reduce the fasting blood sugar level and glycosuria of moderately severe diabetic patients (121).

SALICYLATES

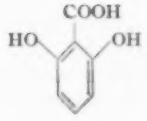
Salicylic acid is known to chelate metal ions and stability constants for salicylic acid with a number of metals have been recorded (122–124). Some specificity for metal-binding under biological conditions may be shown since stability constants with only three metal ions, Fe^{+++}, Al^{+++}, and Cu^{++}, are greater than those of the common amino acids and trace metal ions. Stability constants for a number of salicylic acid derivatives with Fe^{+++}, Al^{+++}, and Cu^{++} ions have also been determined (124, 125).

The fact that the nonchelating *meta* and *para* hydroxybenzoic acids are ineffective in relieving cases of rheumatic fever (126, 127) may be an indication of the importance of metal chelation in the corresponding action of salicylic acid. This has led to the postulation that the classical actions of salicylic acid may be exerted through its chelating function (4). Bergel (128) has also attributed the beneficial antirheumatic effects of salicylates to a reduction *in vivo* of free heavy metal ions, presumably through chelation. In regard to acetylsalicylic acid it has been found in our laboratory (unpublished observation) to form a stable complex with Cu^{++} ion but a stability constant for this reaction has not been obtained.

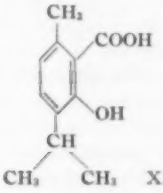
Schubert, *et al.* (129), have examined a variety of salicylates, phenols, catechols, and related structures for their ability to relieve acute beryllium poisoning. Only those compounds capable of chelating beryllium *in vitro* were found to be effective antidotes. It is interesting that the sulfonated derivatives were too water soluble for cell penetration and were ineffective, even though they were able to chelate beryllium. It was also found that formation of an insoluble beryllium chelate *in situ*, by means of aurin tricarboxylic acid (XIX), was as effective in removing the toxic effects of beryllium as attempting to remove the metal from the cells (130).



XIX



XX

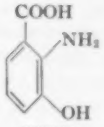


XXI

Cochran, *et al.* (126), found γ-resorcylic acid (XX) to be nearly ten times as effective as salicylic acid in relieving rheumatic fever patients. This increased effect was attributed to a greater degree of chelation by the resorcylic acid but no stability constants were listed. A greater analgesic effect has been reported by O'Brien and and Thoms (131) from a dialkyl-substituted derivative, *o*-thymotic acid (XXI), than from acetylsalicylic acid, and unpublished determinations in our laboratory have shown *o*-thymotic acid to have a lower affinity for Fe^{+++}, Al^{+++}, and Cu^{++} ions than does salicylic acid. A salicylic acid ring substituted by hydroxyl, namely gentisic acid, was also found to have less affinity for metal ions than salicylic acid (124) so it appears that strength of metal-binding is not an important criterion for salicylate actions.

CARCINOGENS AND ANTICANCEROUS COMPOUNDS

β-Naphthylamine, long known to be a potent carcinogen, was shown not to exert this effect (in producing bladder tumors in mice) until it had been metabolized to the *o*-hydroxy derivative, a potent chelator (132). A correlation between the ability to form silver complexes and the carcinogenicity of polycylic aromatic compounds has also been observed (133). Other chelating agents which have been found by Boyland and Watson (134) to produce bladder tumors include the aminonaphthols, 8-hydroxyquinoline, and anthranilic acid [after oxidation to the 3-hydroxy derivative (XXII) *in vivo*]. It was postulated that their carcinogenic effect was due to metal chelation.



XXII

A variety of metal chelates as well as chelating agents have been tested for their effects on the growth of tumors and, although some success has been claimed in slowing or halting this growth, the significance of the results is not yet clear. A résumé follows of some of the more recent attempts. Banga, *et al.* (135), prepared and tested on Ehrlich's tumor in mice a variety of metal complexes of ascorbic acid, citric acid, histidine, malic acid, oxaloacetic acid, and pyrocatechol. The Fe-ascorbic acid complex increased the rate of tumor growth whereas the Cd derivative inhibited growth. Complexes of Mn, in general, were growth inhibiting. All of the growth-inhibiting complexes were too toxic for therapeutic use.

Zinc and cadmium complexes of phosphoriboflavin were claimed by Anichini (136) to have a curative effect on benzopyrene carcinoma in rats. Riboflavin-5′-phosphate has been shown to form metal complexes, which are probably chelates, with divalent Fe, Cu, Co, Ni, and Zn (137). These complexes, in most cases, were highly insoluble in water. Another series of water-insoluble complexes, namely the Fe, Cr, and Cu complexes of the azo coupling products of *o*-aminophenol with α- and β-naphthol, proved to be without effect on the growth of sarcoma 180 in mice in nontoxic doses (98).

A copper-chlorophyll complex has been reported to be somewhat effective in prolonging the life of mice with Ehrlich ascites tumor (138) whereas a mercury-hematoporphyrin was ineffective. A series of oximes, including dimethylglyoxime and some α-keto acid oximes, were found more effective *vs.* Ehrlich carcinoma and Crocker sarcoma in mice when in the presence of Cu and Fe ions (139). Dimethylglyoxime did not exhibit antitumor activity by itself but did in the presence of Cu^{++}. It was inactive, however,

in the presence of Co, Ni, Zn, Fe, Mn, Mg, and Hg ions. Both Cu-dimethylglyoxime and Cu-biacetylmonoxime were also found to inhibit dehydrogenase activity in the carcinoma cells (140), but only the former was active *in vivo*. It was proposed by Takamiya that chelation increased the permeability of the oximes to the cell membranes.

Prolonged administration of α-benzoin oxime as well as dithiocarbamates, in rabbits, had led to destruction of myelin, decrease of the Cu level in blood and serum, and formation of lesions, however (141). These effects were attributed to inhibition of copper proteins.

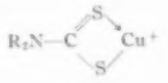
FUNGICIDES

Several fungicides offer well-defined examples of the action of metal chelates *per se*. Reference to the requisite for cupric ion in the fungicidal action of 8-hydroxyquinoline (29, 30) has already been made. For use as a fungicide the copper chelate is employed (142, 143).

The fungicidal action of sodium dimethyldithiocarbamate and tetramethylthiuram disulfide was shown by Goksøyr (144) to depend on the formation of 1:1 complexes with Cu or Zn ions. These complexes were found by spectrophotometric means to bind with proteins, and succinic oxidase was inhibited by the Zn complexes but was unaffected by the Cu complexes. It was concluded that the dimethyldithiocarbamate complexes acted as fungicides by combining with groups in the cell membranes and inhibiting local enzyme systems, probably by competing with coenzyme A or other thiols.

The mode of action of the fungicidal chelating agents has been intensively studied by Janssen and his group. Stability constants and solubility products of the copper complexes of the dialkyldithiocarbamic acids have been determined (145, 146) and the growth-inhibition patterns of these complexes were compared with those of oxine and 2-pyridinethiol-N-oxide copper complexes against *Aspergillus niger* (147) and *Glomerella cingulata* (148). The inhibition patterns were similar, indicating a similar mode of action to that already described for oxine (26) in which both the 1:1 and 1:2 complexes play a decisive role. It was also shown that the activity of higher homologs of dimethyldithiocarbamic acid was limited because of the insolubility of the 1:2 complexes (149). The suggestion was made that these complexes act by interference with a function of lipoic acid.

Dimethyldithiocarbamic acid is used as a fungicide in the form of its sodium salt or zinc or iron complex. The structure of the 1:1 dithiocarbamate chelates has been determined by Chatt, *et al.* (150), to be a four-membered ring, and the 1:1 copper chelate is shown below (XXIII).



XXIII

Other examples of chelating agents which have shown fungitoxic action have been noted by Horsfall and Rich (151) in the case of di-*o*-hydroxy and di-*o*-amino diphenyl sulfides. Other metal chelates with fungicidal properties include copper complexes of variously-substituted salicyl aldehydes and their imines (152) and a number of metal chelates of 2-heptadecylimidazoline (153). Some aromatic dithiocarbamates and their copper complexes showed little antifungal activity (154).

OTHER COMPOUNDS

Cases Where Chelation May be Involved.—*Glucogenic Corticosteroids.*—Some evidence of metal-binding by the glucogenic corticosteroids has been reported. ACTH, for instance, was found to bind Zn^{++} and Cu^{++} ions by use of a polarographic procedure, and a relationship was claimed to exist between the extent of binding and the adrenocorticotropic activity (155). Ions of Co, Ni, and Mn were also bound but the degree of binding showed no relation to bioactivity.

The effect of Au, Bi, Cu, and Hg ions on experimental arthritis in rats has been compared with the action of ACTH and cortisone (156). Therapeutic results and the rate of urinary glucocorticoid excretion following administration of Au and Bi were similar to the effects of the steroids themselves, Cu had a lesser effect, and Hg showed no effect.

The chelating action of cortisone, hydrocortisone, prednisone, and prednisolone has been demonstrated by light absorption and electrical conductance methods (157). Cu and, to a lesser extent, Fe ions were complexed but Co, Ni, Zn, and Mn ions were not. Daily injections (for five days) of these steroids in rats reduced the Cu contents of skin and muscle and increased the amount of Cu in the kidney. Too much importance cannot be placed on the chelating abilities of these steroids, however, until stability constants for the reactions with the trace metals are known, and experiments are conducted in the presence of known amounts of metal ions, an obviously difficult task.

Antiviral Compounds.—Antiviral effects by metal chelates or chelating agents have not been numerous. The cupridihydroporphyrins have shown anti-infectivity action on the hemagglutinating viruses (158), however, and a similar inhibition of both influenza virus infectivity and hemagglutination was postulated for the antiviral action of the benziminazoles (159). The cobalt chelate of ethylenediaminetetraacetic acid (EDTA) was found to exert a definite but transitory suppressive effect against influenza A and B; neither $CoCl_2$ nor EDTA alone showed this effect (160). The inhibitory effects on the larger viruses shown by some thiosemicarbazones (161, 162) could possibly be connected with metal-binding since the thiosemicarbazones are known to chelate (91, 92).

Agents Affecting Transmission at Neuromuscular Junctions.—Charged complex cations of common chelating agents show some of the same biological effects as organic cations. Dwyer, *et al.*, have reported some interesting examples of this by finding inhibition of cholinesterase by metal complexes of *o*-phenanthroline, dipyridyl, and terpyridyl (163), and curariform activity with metal complexes of *o*-phenanthroline, ethylenediamine, and dipyridyl (164). The larger complex ions resembled *d*-tubocurarine in their potency as blocking agents while the smaller ions showed greater similarity to decamethonium ion. The neutral complexes showed none of these effects.

Antiradiation Compounds.—One of the theories which has been suggested regarding the manner in which ionizing radiation damages cells concerns the propagation of hydroxyl radicals by means of a chain reaction, catalyzed or initiated by traces of iron or copper (165). It is interesting that some of the most effective agents yet found to combat radiation damage are powerful chelating agents. The two most notable examples are 2-mercaptoethylamine (166) (XXIV), and 2-mercaptoethylguanidine (167) (XXV). Although stability constants with iron or copper have not been reported for these compounds they would undoubtedly be high.

$$HS{-}CH_2{-}CH_2{-}NH_2$$

XXIV

$$HS{-}CH_2{-}CH_2{-}NH{-}\overset{\overset{NH}{\|}}{C}{-}NH_2$$

XXV

Some evidence for the idea that chelating agents might interrupt the propagation of hydroxyl radicals by sequestering iron or copper might be seen in the findings of Corradi, *et al.* (168). Several chelating agents were found to have a greater protective effect for irradiated mice than did 2-mercaptoethylamine when administrated in equivalent fractions of the toxic dose. Specifically, these agents are diethyldithiocarbamic acid, N-nitroso-N-phenylhydroxylamine, and nitrilotriacetic acid.

Deoxyribonuclease plays a part in the destruction of deoxyribonucleic acid that follows irradiation (169) and it was believed by Bacq, *et al.*, that since this enzyme requires Mg^{++}, complexing agents should have a protective effect against radiation. Citrate failed to protect but fluoroacetate had a protective action in mice after conversion to fluorocitrate, a competitive inhibitor for aconitase. This evidence suggested that a reduction of free Mg^{++} by complexation must be completed at the time of irradiation in order to explain the protective action observed.

Another example where a chelation effect is possible may be seen in the radiation-protection afforded by 3-mercaptomethyl-4-hydroxymethyl-5-hydroxy-6-methylpyridine (170), a thio analog of pyridoxine which is capable of metal chelation. 3-Mercaptomethylpyridine, a nonchelating relative, had no protective effect on mice.

Miscellaneous.—Both antithyroid compounds, such as thiouracil, and cardiac glycosides have been shown to cause reduction of tissue metal levels (171). In this connection cobaltous chloride produces a hypothyroid effect which may be due to reaction with thyroxine (172). Wilbrandt (173) has postulated that cardiac glycosides act as competitors for chelates presumably formed by corticosteroids. The beneficial effect of zirconium salts in treating poison ivy (*Rhus toxicodendron*) dermatitis has been attributed to chelation of the catechol groups in urushiol (174) the toxic principle (175).

Cases Where Chelation Is Probably Not Involved.—Weinberg (16) has called attention to a variety of compounds capable of chelation which have no antimicrobial activity and Albert (32) and Schraufstätter (176) have also pointed out that the majority of the spot test reagents, notoriously powerful chelating agents, are without antibacterial properties. Evidence is also plentiful that the majority of antitubercular substances are chelating compounds but most chelating compounds are not antitubercular (137).

Epinephrine is a molecule with a chelating function, the catechol moiety, and chelation with metals does occur (177, 178). The chelate with boron did not alter its physiological potency (177), however. Other chelating and complexing substances potentiate the action of epinephrine,

such as EDTA (179), cysteine, cystine, ascorbic acid, and tyrosine (180), flavonoids, 8-hydroxyquinoline, and diethyldithiocarbamate (181), trihydroxy-N-methylindole (182), salicylates (183), and 1-hydrazinophthalazine (184). It appears likely to most investigators that these potentiators act by chelating copper or iron and presumably lowering or preventing its ability to catalyze the destruction of epinephrine.

The sensitization of smooth muscle to adrenaline that is caused by many plant phenolics containing the catechol nucleus has been attributed to a reversal of inhibition of methyltransferase (185). Addition of catechol to methyltransferase solutions inhibited by ATP or methionine removed the inhibition and could well be a reflection of the ability of catechol to chelate metal ions needed for the enzyme activity or that interfere with the action of adrenaline.

Some purines, notably the 6-hydroxy derivatives, are capable of chelating metals but it is unlikely that chelation is involved anywhere in the stimulatory action of the purines. While theophylline chelates copper (186), caffeine and 6-desoxytheophylline are unable to, and all these compounds have similar effects in stimulating the hypodynamic heart-lung preparation (187).

Histamine chelates metals (188, 189) and ferrous ion has an inhibitory effect on histamine action *in vivo* (190) but it appears likely that metal chelation is an unwanted or competitive reaction only. The Cu^{++} complex causes a weaker contraction of guinea pig ileum than histamine itself (189). Some agreement has been found between assumptions and evidence that histamine acts as a carrier for sodium ion, with other metal ions acting as competitors (173).

DISCUSSION AND SUMMARY

It should now be evident that convincing proof that metal chelation is an essential step in the mechanism of action of chelating drugs is an elusive goal. Whereas in the majority of cases where chelation is implicated the evidence is not complete, neither is it probable that the establishment of chelation mechanisms will offer a complete explanation of chelating drug activities. However, the total accumulation of evidence regarding chelation as one step in the mechanism of a number of drugs is now sufficiently great to establish it as a likely possibility where chemical studies show that metal chelation is possible.

Regarding the possible ways in which a chelating agent might be involved *in vivo*, previously outlined, the following examples might be offered as support.

Transient combination of a chelator with a metalloenzyme is possible with agents affecting sugar metabolism and the salicylates. A direct combination of salicylic acid with a copper enzyme, tyrosinase, has been observed in our laboratory, for instance (unpublished observation).

Removal of metal ions from the cellular environment which deprives an organism of its trace metal requirement has been postulated in regard to the action of antibacterials and fungicides, (24, 42, 43, 47, 55, 80) but no evidence of such a process has been provided. Removal of toxic cations in cases of metal poisoning has been reported, however (13–15).

The utilization of metal chelation as a mechanism of transport of drugs through semipermeable membranes is a possibility in the cases of *p*-aminosalicylic acid (86) and isoniazid (74). This possibility has been recently discussed by Wilbrandt (173) as a basis for certain pharmacological actions of histamine, the cardiac glycosides, and corticosteroids.

Stabilization of a molecule by chelation of metals which catalyze its destruction has been observed in the protective effects of chelators for epinephrine (179–184). Stabilization by combination with metal ions could also explain the increased blood sugar effects observed for alloxan (108) which is converted to alloxanic acid in neutral or alkaline media.

Examples where the formation of an "unsaturated" chelate (one where the metal ion is still capable of coordination with another ligand molecule) exerts a biological action can be seen in the antibacterial effect of 8-hydroxyquinoline (26), and the fungicidal effect of dimethyldithiocarbamic acid (144, 149).

No documented cases have appeared where chelation by a drug has been found to alter the oxidation potential of a metal. It is a possibility in regard to the effects of some antiradiation compounds, however (166, 167); and the reversals of activity of some drugs shown by cobaltous ion, as in the case of 8-hydroxyquinoline (26), could also involve this phenomenon.

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Investigation of Methyl Pyridinium-2-aldoxime Salts

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Methyl pyridinium-2-aldoxime iodide (2-PAM iodide) has been reported to be effective in preventing and overcoming the toxic effects in animals poisoned with organic phosphorus compounds that are inhibitors of the cholinesterase enzymes. In order to obtain greater water solubility of the oxime and to eliminate undesirable side effects due to the iodide ion, various salts were prepared. The iodide ion was replaced by inorganic and organic ions: chloride, nitrate, dihydrogen phosphate, hydrogen sulfate, acetate, lactate, fumarate, and tartrate. Methods of preparation and physical contents of the salts are presented. The water solubility of the majority of these salts was many times greater than that of the iodide. The rate at which the various salts reactivate inhibited cholinesterase and the acute toxicity to mice are of the same order as that of 2-PAM iodide. On the basis of stability, physical properties, and pharmacological action, the 2-PAM chloride salt should be the one of choice.

A GROUP of highly toxic organic phosphorus compounds, which includes some of the most potent chemical warfare agents (1), is finding increasing use in agriculture as insecticides. Found in this group are parathion (O,O-diethyl-O-nitrophenyl thiophosphate), TEPP (tetraethyl pyrophosphate), HETP (hexaethyltetraphosphate), OMPA (octamethyl pyrophosphoramide), Systox (a mixture of O,O-diethyl-O-ethylthioethyl phosphorothionate and O,O-diethyl-S-ethylthioethyl phosphorothiolate), Sarin (isopropylmethyl phosphonofluoridate), and Tabun (ethyl phosphorodimethylamide cyanidate). These compounds, or compounds resulting from biotransformation, produce their toxic effects mainly by inhibiting the cholinesterase (ChE) enzymes throughout the body (2). The resulting abnormal accumulation of acetylcholine has the following consequences: it results in muscarinic effects at the ends of postganglionic cholinergic nerves causing nausea, vomiting, abdominal cramps, diarrhea, sweating, increased salivary and bronchial secretions, and bradycardia; at the ends of motor nerves to skeletal muscle nicotinic effects are produced, e. g., weakness and fasciculations; in the central nervous system anxiety, headache, and possible ataxia, coma, and convulsions result (3–5). Severe poisoning by these compounds may result in paralysis of the muscles of respiration and death.

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Among the many drugs that have been studied in an attempt to block or overcome the toxic action of acetylcholine, atropine has been the most satisfactory and is the only drug presently standard for the treatment of poisoning by anticholinesterases or the compounds commonly referred to as nerve gases (5). Although atropine is effective in counteracting the muscarinic effects of acetylcholine, it is severely deficient in that it cannot overcome the neuromuscular block causing paralysis of the muscles of respiration.

A number of hydroxamic acids and oximes have been found to regenerate cholinesterase activity *in vitro* after the enzyme had been inactivated by organophosphate anti-ChE compounds (6–9). One oxime, methyl pyridinium-2-aldoxime iodide, also designated as pyridine-2-aldoxime methiodide but commonly referred to as 2-PAM,[1] was found to be a very rapid reactivator *in vitro* of inactivated ChE (10, 11). In combination with atropine the oxime has been found to be effective in reducing the toxicity of ChE-inactivating phosphates (12–17) in various animal species and to have clinical value in the treatment of poisoning by these toxic chemicals (18, 19).

In man, the intravenous dose of 2-PAM iodide required to alleviate the general weakness caused by an anti-ChE chemical, such as Sarin, is 1 to 2 Gm. (18), which is equivalent to 14–28 mg./Kg. The solubility of 2-PAM iodide has been reported to be of the order of 20 mg./ml. of water at room temperature (16), on the basis of which an injec-

[1] It is suggested that the term 2-PAM be used to designate the oxime moiety, and that the various salts be referred to as 2-PAM iodide, 2-PAM chloride, etc.

tion of 50 to 100 ml. would be required to administer an effective amount of the antidote. This volume may be given intravenously, but under field conditions intramuscular administration would be desirable for emergency treatment of poisoning caused by anti-ChE agents. The intramuscular injection of 50–100 ml. of solution would be impracticable. Askew stated that 2-PAM iodide could not be tested by intramuscular injection in large animal species, such as the monkey, because of the large volume of solution required (16). Another disadvantage is that iodism may result from the administration of large amounts of 2-PAM iodide since approximately 50 per cent, by weight, of the molecule is iodide ion. In order to overcome the deficiencies of the iodide salt, other salts were prepared in which the anion was chloride, nitrate, hydrogen sulfate, dihydrogen phosphate, fumarate, tartrate, or lactate. Of these salts the chloride has been prepared (20), but no reference to solubility was given.

The statement has been made that the anionic part of the oxime salt is not critical (21). This view is acceptable on the basis of the evidence that the therapeutic value of the salt is a function of the quaternary oxime moiety. However, the anion used to form the salt can confer physical properties of importance and significance for the formulation and administration of the compound.[2]

EXPERIMENTAL

Preparation.—The oxime salts were prepared by the following general procedures. The silver salt and 2-PAM iodide were each dissolved in minimal volumes of water and mixed. The amount of 2-PAM iodide taken was slightly in excess of that required for stoichiometry. After the mixture was shaken for thirty minutes, the silver iodide was filtered and the filtrate evaporated to dryness with a steam bath and reduced pressure, using a Rinco rotating evaporator. The residue was recrystallized from 95% ethanol.

The formation of the hydrogen sulfate salt was brought about by reacting silver sulfate, dissolved in 0.1 *N* sulfuric acid, with 2-PAM iodide. The above procedure was followed for isolation and purification of pyridine-2-aldoxime methyl hydrogen sulfate. For the dihydrogen phosphate salt, silver *ortho*-phosphate was dissolved in a minimum volume of 85% phosphoric acid, diluted 1:15 with water, and added to an aqueous solution of 2-PAM iodide. After filtering off the silver iodide, an equal volume of ethyl alcohol was added to the filtrate. Addition of an excess of ethyl ether precipitated crystalline pyridine-2-aldoxime methyl dihydrogen phosphate.

The chloride and lactate salts were also prepared by means of ion exchange resins, using the column technique. For the lactate salt, Dowex-1-chloride resin was converted to the hydroxyl form by treatment with a concentrated sodium hydroxide solution. After washing the column free of alkali, an excess of lactic acid was passed through the column to convert the resin to the lactate form. The column was again washed with water until free of acid. An aqueous solution of 2-PAM iodide, in an amount equivalent to 75% of the capacity of the resin lactate, was added to the column through a separatory funnel and the column washed with distilled water. The eluate, which was free of halide, was collected, evaporated to dryness, and the lactate salt isolated as previously described.

The chloride salt was prepared in a similar manner, using two strong anionic exchange resins: Rohm and Haas Amberlite 1RA-400 and Dowex-1. A molar solution of sodium chloride was passed through both columns and the resins then washed with water until free of halide. Solutions of 2-PAM iodide were added and the columns washed with water as previously described. The fractions of the eluate containing halide were collected and the chloride salt isolated as described.

The lactate salt has a high water solubility, is hygroscopic, and recrystallizes with difficulty when moist. Therefore, an alternate isolation procedure was devised in which the oxime was dissolved in absolute ethanol and the silver lactate added in small increments to the solution, with constant stirring. The mixture was stirred for thirty minutes in a stoppered flask, and the precipitated silver iodide filtered and washed with cold absolute ethanol. The filtrate was evaporated to dryness under reduced pressure on a water bath maintained at 30–35°. The residue was triturated with absolute ether and filtered in a humidity controlled cabinet or by drawing a stream of dry air through the Büchner funnel. The precipitate was transferred to a desiccator and dried *in vacuo*. Analytical and physical data of the salts are given in Table I.

Reactivation Rates of Inhibited Eel Cholinesterase.—The apparent rates of reactivation of eel cholinesterase, completely inactivated with a slight excess of a potent anticholinesterase compound, were determined by a titrimetric procedure in which the substrate, acetylcholine, was present in the reaction mixture of the oxime and inhibited enzyme.[3] The results, given in Table II, show no significant differences among the apparent reactivation rates.

Stability.—In a study of the stability of 2-PAM salts, using the techniques of chemical kinetics, the observed rate of deterioration of the oxime at constant pH and temperature was shown to be first order with respect to drug concentration (23). Also, two pathways were postulated for the breakdown of the oxime: below approximately pH 4 hydrogen ion catalysis of the acid form of the oxime occurs; above this pH the reaction is either a hydroxyl ion catalysis of the acid form or a direct attack on the oximate species, i. e., noncatalytic. The hydrogen ion concentrations of maximum stability were found to vary from pH 4.93 at 0° to pH 3.20 at 100°.

On the basis of the stability data, aqueous solu-

[2] While these studies were in progress the preparation of the methane sulfonate salt was reported (22).

[3] We gratefully acknowledge our indebtedness to Dr. Harry O. Michel for carrying out these reactivation rate studies.

TABLE I.—ANALYTICAL AND PHYSICAL DATA OF METHYL PYRIDINIUM-2-ALDOXIME (2-PAM) SALTS

2-PAM Salt	Empirical Formula	Analysis: Carbon Calcd., %	Carbon Found, %	Hydrogen Calcd., %	Hydrogen Found, %	Neut. Equiv. Calcd., %	Neut. Equiv. Found, %	M. P. ° C.	Solubility mg./ml. of Soln. at 25°
Chloride	$C_7H_9N_2ClO$	48.7	48.6	5.3	5.2	...	...	226–227 (decompn.)	640
Nitrate	$C_7H_9N_3O_4$	42.2	42.6	4.5	4.4	199	196	155–156 (decompn.)	675
Dihydrogen phosphate	$C_7H_{11}N_2O_5P$	35.9	35.7	4.7	4.7	234	239	214–215 (decompn.)	46
Hydrogen sulfate	$C_7H_{10}N_2O_5S$	36.2	35.9	4.4	4.3	235	236	164–165 (decompn.)	640
Iodide	$C_7H_9N_2IO$	31.8	32.0	3.4	3.5	264	267	225–226 (decompn.)	48
Fumarate	$C_{18}H_{20}N_4O_8$	55.7	54.0	5.2	4.0	194	196	168–169 (decompn.)	389
Acetate	$C_9H_{12}N_2O_3$	55.1	52.3	6.2	6.6	...	...	106–107 (decompn.)	...
Tartrate	$C_{18}H_{22}N_4O_9$	49.2	51.1	5.5	5.3	221	235	158–160 (decompn.)	565
Lactate	$C_{16}H_{14}N_2O_4$	53.0	53.1	6.2	6.3	226	224	115–116 (decompn.)	1,000

TABLE II.—APPARENT RATES OF REACTIVATION OF INACTIVATED EEL CHOLINESTERASE BY METHYL PYRIDINIUM-2-ALDOXIME (2-PAM) SALTS

2-PAM Salt	Concn., *M*	Apparent Reactivation Rate Constant—Liter Mole^{-1} min.$^{-1}$
Iodide	1.48×10^{-4}	1.03×10^{3}
Nitrate	1.16×10^{-4}	1.23×10^{3}
Hydrogen sulfate	1.47×10^{-4}	1.08×10^{3}
Fumarate	5.62×10^{-5}	1.87×10^{3}
Tartrate	9.6×10^{-5}	1.52×10^{3}
Lactate[a]	2.5×10^{-4}	8.3×10^{2}
Iodide[a]	2.5×10^{-4}	8.4×10^{2}

[a] Determinations were carried out at a time different from that of the other salts.

tions of 2-PAM salts should undergo autoclaving without significant breakdown, provided a suitable pH is maintained. Unbuffered 2.5 (isotonic), 10, and 20% solutions of the chloride salt, of pH 4.3, 3.9, and 3.5, respectively, autoclaved at 120° and at 15 lb./15 min. showed a loss of only 4% of oxime, as determined by ultraviolet spectrophotometry (24).

Cyanide ion has been reported to be a decomposition product of 2-PAM (25). If the cyanide is produced stoichiometrically, then 100 mg. of the chloride salt would produce only 0.6 mg. of cyanide. Furthermore, 10 and 20% solutions of 2-PAM chloride that were stored in ovens at 50 and 70° from one to three months, showed a minimum oxime content of 78%, whereas the maximum cyanide content, as determined by the method of Aldridge (26), was 0.4%. Selected samples of these stored solutions were injected into mice and the systemic toxicity compared to that of a freshly prepared solution of the chloride salt. The results indicated that the toxicity of the stored samples was due only to the oxime content.

From the above experiments it is clear that aqueous solutions of salts of 2-PAM can be sterilized by autoclaving and stored for reasonable periods of time without deleterious effects.

Toxicity.—A comparison of the acute toxicity of 2-PAM iodide with that of the various water-soluble salts was made by intraperitoneal injection into mice of 16 Gm. average weight.[4] Aqueous solutions of the compounds were injected at the rate of 10 ml. per Kg. The period of observation was four hours; the animals that died did so usually within thirty minutes. The LD_{50} of 2-PAM iodide was found to be 220 mg./Kg. (0.83 m*M*/Kg.), with 19/20 C.L. of 207–234 mg./Kg. The toxicity of the water-soluble salts on a molar basis was not significantly different from that of 2-PAM iodide with the exception of the dihydrogen phosphate salt, which was approximately 15% more toxic.

[4] We are indebted to Mr. P. Zvirblis for his valuable assistance with the bioassays.

DISCUSSION

The pharmacological properties of the anion as well as the physical properties of the oxime salt itself must be considered in the selection of a salt of 2-PAM for use in the prophylaxis and therapy of anticholinesterase poisoning owing to the relatively large amount of oxime that may be required. Accordingly, the following information relative to the pharmacology of the anions used in this study is of interest (27).

Nitrate salts are irritating to the gastrointestinal tract, causing nausea and gastric distress. Furthermore, under the influence of intestinal bacteria, nitrate ion may be converted to nitrite, which oxidizes hemoglobin to methemoglobin. Both bromide and iodide ions are known to produce disturbances of the skin, as part of the general effects described as bromidism and iodism, respectively. Absorption of the sulfate ion from the gastrointestinal tract is slight and sulfate salts, therefore, may act as saline cathartics. With regard to the organic anions, the tartrate ion is absorbed with difficulty from the gastrointestinal tract; however, a high concentration of tartrate ion in the blood, such as may occur after parenteral administration of a tartrate salt, is known to cause renal damage. The citrate ion, an intermediate in carbohydrate metabolism, forms a soluble complex with calcium that is poorly dissociable. The danger of citrate intoxication, however, is remote. The acetate and lactate ions are normal metabolites having no specific pharmacological actions and both appear to be well tolerated in relatively large amounts. No data on the pharmacology of the fumarate ion were found.

The salts listed in Table III, with the exception of the dihydrogen phosphate, showed a marked and significant increase in water solubility over that of the iodide, the lactate salt being the most soluble. On the basis of the data, approximately 1 Gm. of

TABLE III.—PERCENTAGE OF OXIME MOIETY IN METHYL PYRIDINIUM-2-ALDOXIME (2-PAM) SALTS

2-PAM Salts	Percentage Oxime	Molar Ratio[a]
Chloride	79.5	1
Nitrate	68.9	1.15
Dihydrogen phosphate	58.6	1.36
Hydrogen sulfate	58.6	1.36
Iodide	51.9	1.53
Fumarate	70.6	1.13
Acetate	69.9	1.14
Tartrate	64.9	1.22
Lactate	60.6	1.31

[a] Ratio of molecular weight of 2-PAM salt to that of the chloride salt and represents the weight of the salt that contains an amount of oxime equivalent to that in 1 Gm. of 2-PAM chloride.

the water-soluble salts could be given parenterally in a volume of 2–3 ml. Thus, the rapid intramuscular injection of large amounts of oxime becomes practicable for large animals and man.

The anions would not be expected to influence the stability of solutions of salts of 2-PAM since the reactions involved in the breakdown of the oxime are governed mainly by pH and temperature of the solutions. The slight deterioration that occurred on autoclaving solutions of salts of 2-PAM was in agreement with values predicted on the basis of kinetic data obtained at various temperatures and pH values.

In the solid form the lactate and acetate salts of 2-PAM were markedly unstable. After two weeks at ambient temperature the acetate salt underwent noticeable decomposition, which progressed to the production of a tarry mass; deterioration of the lactate salt set in after four to six weeks and progressed to the formation of a dark brown color. The other salts were stable at room temperature and showed no significant loss of oxime content after two to three months at 50°.

The percentage of oxime contained in a salt of 2-PAM is highly important if the compound is to be taken orally as a tablet or capsule for prophylactic purposes. In this situation the largest weight of oxime per weight of solid item would be advantageous, especially in view of the relatively large amount of oxime that may be required. Table III shows that the chloride and nitrate of the inorganic salts and the fumarate and acetate of the organic salts are superior in this respect. The chloride salt, with 79.5%, contains the most oxime per unit weight of salt [b]

The chloride salt of 2-PAM on the basis of physiological compatibility, water solubility, stability, and percentage of oxime contained, should be the one of choice for use in the therapy and prophylaxis of anticholinesterase poisoning.

The preparation of 2-PAM chloride from pyridine-2-aldoxime and methyl chloride is under investigation and will be reported elsewhere.

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[b] The methane sulfonate salt of 2-PAM contains only 56.6% of oxime; 1.4 Gm. is equivalent in oxime content to 1 Gm. of the chloride salt.

Stability of Aqueous Solutions of N-Acetyl-*p*-aminophenol

By K. THOMAS KOSHY and JOHN L. LACH

The hydrolysis of N-acetyl-*p*-aminophenol (NAPAP) in hydrochloric acid solutions of varying strengths at 70° and in buffered aqueous solutions in the pH range of 2 to 9 has been studied. The hydrolysis appears to be hydrogen and hydroxyl ion catalyzed. The overall rate of hydrolysis may be expressed by the equation $k_H \cdot (H^+)(NAPAP) + k_{OH} \cdot (OH^-)(NAPAP)$. The energy of activation for the hydrolysis was calculated to be of the order of 17.5 Kcal. per mole which is in good agreement with that reported for similar compounds.

NUMEROUS COMPOUNDS which are important from a pharmaceutical standpoint contain an amide function, yet there have been few quantitative studies concerning their hydrolytic stability. This lack has perhaps been due, in part, to the relative stability of amides, as compared for example, with the structurally similar esters. However, a number of studies have been reported on the rate and mechanism of hydrolysis of aliphatic and aromatic amides in general. Most of these studies have been conducted in strong acids or bases. The rates of the acid catalyzed hydrolysis of several amides have been reported to go through a maximum (1–5). The effect of substituents in the acid and base catalyzed hydrolysis of amides have been reported by Remsen (6), Reid (7, 8), and Maloche and Laidler (9).

The rates of hydrolysis of anilides as compared to that of amides would be expected to be different, since in anilides the basicity of the nitrogen is weakened by substitution of the benzene ring. The effect of substituent groups in anilide hydrolysis is in accordance with the electronic theory of valency. This has been demonstrated by Karve and Kelkar (10) in their study of the acid hydrolysis of a few anilides including acetanilid. N-Acetyl-*p*-aminophenol (NAPAP) is a substituted anilide, but no information is available on the hydrolytic rate of this compound. Since NAPAP has recently gained considerable pharmaceutical importance, as evidenced by the number of preparations containing this compound, it was felt that data concerning the kinetics of this compound would be of value. The purpose of this study, therefore, was to investigate the kinetics of the degradation of NAPAP in aqueous solutions of various pH.

EXPERIMENTAL

In order to carry out such an investigation, an analytical procedure for the determination of NAPAP or its breakdown products, *p*-aminophenol (PAP) or acetic acid, was necessary. The development of such a method was complicated by the fact that *p*-aminophenol itself is unstable. A procedure was therefore developed to separate NAPAP from PAP by ion exchange chromatography (11). Although complete separation of NAPAP was effected by this procedure, its concentration could not be determined directly by its absorption in the U. V. region because the eluant contained, in addition to NAPAP, the degradation products of PAP which caused interference. The concentration of NAPAP in this solution was therefore determined colorimetrically by the procedure of Greenberg and Lester (12). According to this procedure, NAPAP was hydrolyzed in 2*N* hydrochloric acid to PAP and coupled with an alcoholic solution of α-naphthol in alkaline media to develop a color, the intensity of which is proportional to the concentration of PAP.

Apparatus and Reagents.—N-Acetyl-*p*-aminophenol, Eastman Organic Chemicals, recrystallized m. p. 167–168°; α-naphthol, freshly resublimed, m. p. 95°; concentrated hydrochloric acid; 30% sodium hydroxide solution; ion exchange resin, Amberlite IR-120, Rohm and Haas Co.; chromatographic glass column of approximately 5 mm. diameter; 0.2*M* phosphoric acid buffer (pH 2, 3, and 4), 0.2*M* phosphate buffer (pH 5, 6, 7, and 8), 0.2*M* borate buffer (pH 9); constant temperature bath with Sargent heater, circulator, and regulator; thermometer calibrated to 0.1; Beckman pH meter; Beckman model DU spectrophotometer; and silica cells.

Procedure.—NAPAP, 0.15 Gm., was accurately weighed into a 100-ml. volumetric flask and dissolved in about 90 ml. of the buffer solution ranging in pH from 2 to 9. The buffer solution was preheated to the temperature at which the particular run was made to effect quick dissolution. After the solution was complete the flask was placed in the constant temperature bath and the volume made up to the mark with hot buffer solution and the contents mixed. After a lapse of about five minutes, to allow the solution to equilibrate thermally, 5 ml., representing the zero-hour sample, was withdrawn, transferred to a 100-ml. volumetric flask, cooled to room temperature, and the volume made up to 100 ml. with deionized water. A 15-ml. aliquot of this solution was run through a column of a strong cation exchange resin in the hydrogen form to remove the PAP formed by hydrolysis of NAPAP. Approximately 0.5 Gm. of the resin was used for this operation. After the solution and the washing had passed below the upper level of the resin, the

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column was eluted with deionized water to a volume of 100 ml. A 15-ml. aliquot of this solution was transferred to a 25-ml. volumetric flask and 3 ml. of concentrated hydrochloric acid added. The flask was stoppered and heated on a boiling water bath for forty-five minutes to hydrolyze NAPAP to PAP. After cooling to room temperature, two drops of a freshly prepared 10% alcoholic solution of resublimed α-naphthol was added, followed by 5 ml. of a 30% sodium hydroxide solution. The volume was made up to the mark and the flask heated for exactly one minute in a bath maintained at 90° and cooled immediately to room temperature. Absorbance of this solution was determined at 590 mμ within three to five minutes after development of color. Samples were removed at definite intervals of time and analyzed by this procedure. A standard solution of NAPAP, hydrolyzed to *p*-aminophenol in the manner described, was used to calculate the concentration of NAPAP present in the sample. A separate standard was run with each set of samples as slight variations in absorbance were observed from day to day for solutions containing the same amount of pure NAPAP. This variation is probably due to the instability of the α-naphthol solution used for color development.

pH and Temperature Effect on the Rate of Hydrolysis.—In order to study the effect of pH and temperature on the rate of hydrolysis of NAPAP, the reaction was carried out at three different temperatures in buffer solutions of different pH as follows: pH 2, 3, 6, 8, and 9 at 70°; pH 2, 3, 4, 6, 8, and 9 at 80°; and pH 2, 3, 4, 5, 6, 7, 8, and 9 at 90°. For solutions of pH 2 at 80 and 90°, it was noticed that after hydrolysis had proceeded approximately 50% there was interference in the analytical procedure due to the oxidation products of PAP. Therefore, the reaction at this pH for these temperatures was studied up to forty and sixty-five hours, respectively. For solutions of pH 9 at 80 and 90°, interference was noticed after the reaction had proceeded to approximately 30% completion. Therefore, the reaction at this pH and these temperatures was studied up to sixty-five hours. This interference, however, could be corrected for by developing the color before and after hydrolysis of the eluant containing NAPAP to PAP in concentrated hydrochloric acid, the difference in absorbance of the two solutions representing the concentration of NAPAP. For the rest of the solutions, the reaction was followed up to a minimum of ninety hours.

Procedure for Studying the Effect of Initial Concentration on the Order and Rate of Hydrolysis of N-Acetyl-*p*-aminophenol.—Essentially the same procedure as above was carried out using 0.15 and 0.30% solutions of NAPAP in buffer solutions of pH 2. The reaction was carried out at 90°.

Effect of Ionic Strength on Rate.—The effect of ionic strength on the rate of hydrolysis of NAPAP was investigated by studying the hydrolytic rate at 90° of a solution of NAPAP in 0.2 *M* phosphoric acid buffer at pH 2 containing 1% potassium chloride and comparing this rate with that of another solution of NAPAP of the same concentration in the same media without potassium chloride.

Hydrolysis of N-Acetyl-*p*-aminophenol in Hydrochloric Acid Solutions of Varying Strengths.—Since the rate of hydrolysis of NAPAP was slow even at pH 2 at 90°, it was decided to study the hydrolysis of NAPAP in aqueous hydrochloric acid solutions of varying strengths. For this purpose, 0.15% solutions of NAPAP were prepared in 100-ml. volumetric flasks at 70° in 0.2, 0.4, 0.6, 0.8, and 1.0 *N* hydrochloric acid solutions, respectively. The flasks were kept in a constant temperature bath maintained at 70° and after a lapse of five minutes for thermal equilibration, 5 ml., representing the zero-hour sample, was withdrawn from each flask and transferred to 100-ml. volumetric flasks. The solution was cooled immediately and the volume made up to 100 ml. with deionized water. A 10-ml. aliquot of this solution was run through a column of a strong cation exchange resin to remove the PAP formed by hydrolysis and the column eluted with deionized water to a volume of 100 ml. Absorbance of this solution was determined at 244 mμ, the wavelength of maximum absorbance for NAPAP. The concentration of NAPAP in these samples could be followed by direct spectrophotometric determination after removal of PAP, as there was no interference from the oxidation products of PAP, it being very stable in the strongly acidic medium. Samples were withdrawn at definite intervals of time and analyzed as above.

The effect of concentration on the degradation rate in aqueous hydrochloric acid solution was investigated by studying the rate of hydrolysis of 0.10, 0.15, and 0.20% solutions of NAPAP in 1.0*N* hydrochloric acid at 70°.

RESULTS AND DISCUSSION

The results obtained indicate that the hydrolysis of NAPAP is both an acid and a base catalyzed reaction. Since spontaneous hydrolysis was found to be negligible, the rate equation may be written as

$$\text{rate} = k_{H^+}(H^+)(\text{NAPAP}) + k_{OH^-}(OH^-)(\text{NAPAP}) \quad \text{(Eq. 1)}$$

If the reaction is carried in an acidic solution, hydrogen ions are then the only effective catalyst and the rate equation is reduced to

$$\text{rate} = k_{H^+}(H^+)^n(\text{NAPAP})^m \quad \text{(Eq. 2)}$$

Similarly in an alkaline solution

$$\text{rate} = k_{OH^-}(OH^-)^n(\text{NAPAP})^m \quad \text{(Eq. 3)}$$

Since hydrogen and hydroxyl ions were maintained constant, equations 2 and 3 may be written as

$$\text{rate} = k'_{H^+}(\text{NAPAP})^m \quad \text{(Eq. 4)}$$

and

$$\text{rate} = k'_{OH^-}(\text{NAPAP})^m \quad \text{(Eq. 5)}$$

where

$$k'_{H^+} = k_{H^+}(H^+)^n \quad \text{(Eq. 6)}$$

and

$$k'_{OH^-} = k_{OH^-}(OH^-)^n \quad \text{(Eq. 7)}$$

The hydrolysis is first order with respect to NAPAP, since straight line relationships were obtained when the logarithm of the concentration of NAPAP was plotted as a function of time (Figs. 1–7). Figures

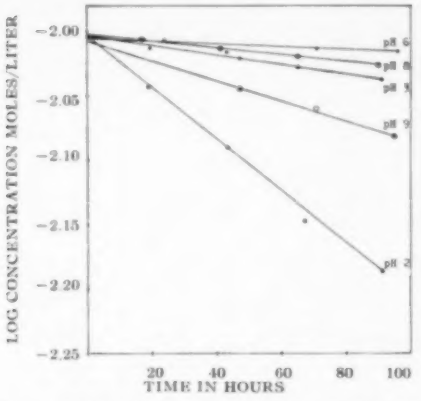


Fig. 1.—Log of the concentration of NAPAP against time at 70° at different pH's.

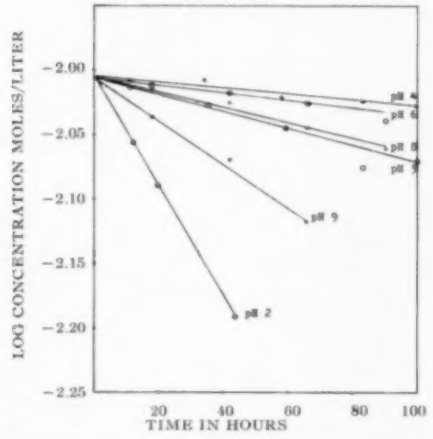


Fig. 2.—Log of the concentration of NAPAP against time at 80° at different pH's.

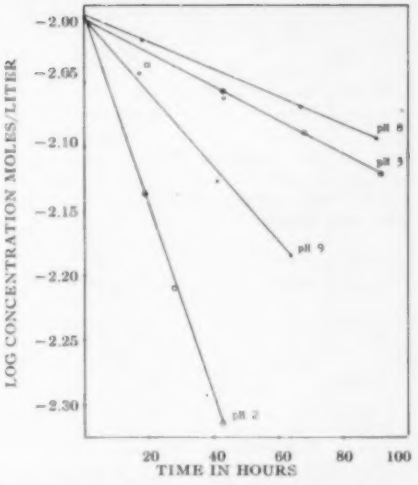


Fig. 3.—Log of the concentration of NAPAP against time at 90° at different pH's.

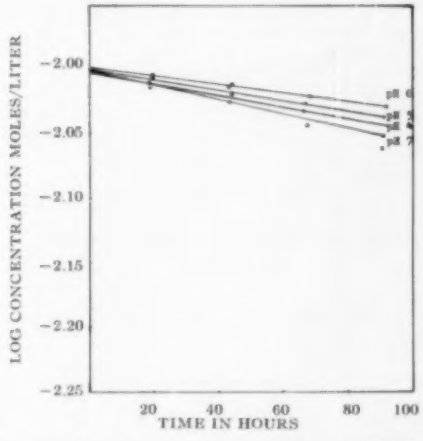


Fig. 4.—Log of the concentration of NAPAP against time at 90° at different pH's.

5 and 7 further demonstrate that ionic strength has no influence on the rate and also that the rate is directly dependent on the concentration of NAPAP.

The order of the reaction with respect to H^+ and OH^- may be obtained from Eqs. 6 and 7. In logarithmic form, these become

$$\log k'_{H^+} = \log k_{H^+} + n \log (H^+) = \log k_{H^+} - n \text{ pH}$$

and

$$\log k'_{OH^-} = \log k_{OH^-} + n \log (OH^-) = \log k_{OH^-} + n \text{ pH}$$

Therefore, plots of log k'_{H^+} and k'_{OH^-} *versus* pH should be a straight line, n, being numerically equal to the slope of the line. Figure 8 which is a plot of log k' *vs.* pH for the hydrolysis in aqueous hydrochloric acid solutions of varying strengths clearly shows that the reaction is first order with respect to hydrogen ion concentration. The pH's of these solutions were determined from their activity coefficients at 70°, calculated according to the procedure outlined by Harned (13). Figure 9 is a similar plot for the hydrolysis in buffered solutions in the pH range of 2 to 9. The slope of the graph in the region of maximum acid hydrolysis, that is pH 2 is very close to −1. The slope of the graph in the region of maximum hydrolysis in the alkaline pH is less than one. This could be due either to experimental error or to lack of data at higher pH values. In alkaline solutions however, it is possible that the phenolic hydroxyl group of the NAPAP molecule may undergo ionization. If this be the case, the rate and mechanism of hydrolysis may not obey simple first-order kinetics and could account for the observed deviation. The dotted line in the

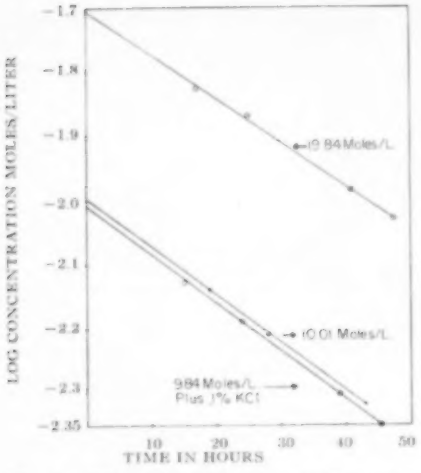


Fig. 5.—Effect of initial concentration and of ionic strength on the hydrolysis rate of NAPAP at 90° in 0.2 *M* phosphoric acid buffer of pH 2.

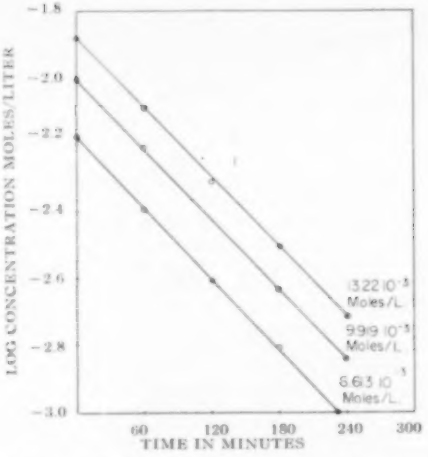


Fig. 7.—Effect of concentration on the rate of hydrolysis of NAPAP in 1.0 *N* hydrochloric acid at 70°.

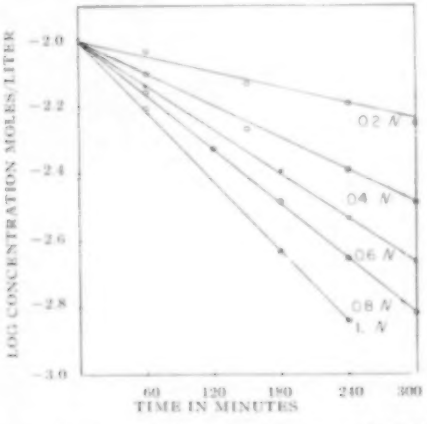


Fig. 6.—Log of the concentration of NAPAP against time at 70° in hydrochloric acid of different concentrations.

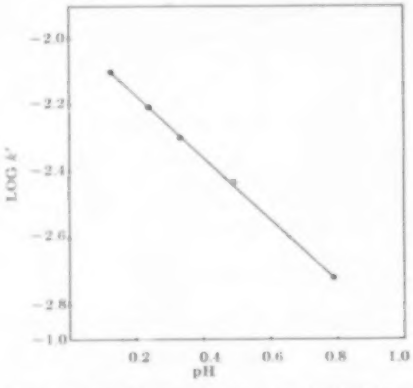


Fig. 8.—Log k' *vs.* pH for the hydrolysis of NAPAP at 70° in aqueous hydrochloric acid solutions of varying strengths showing the first order of the reaction with respect to H^+ ion concentration.

graph is based on theoretical considerations and the assumption that there is no appreciable ionization at the pH's studied. The slope of the graph in this region is one and may be considered as the order of the reaction with respect to hydroxyl ion concentration. The order of the reaction in the pH range 4 to 7 is not first order with respect to either hydrogen or hydroxyl ion concentration but of a complex order.

Specific reaction constants for the hydrolysis of NAPAP in aqueous solutions at various pH's were determined at three temperatures (Table I). The Arrhenius plot of log k' *vs.* $1/T$ was found to give a straight line (Fig. 10), indicating that the mechanism responsible for the hydrolysis is not altered with change in temperatures. The energy of activation calculated from the slopes of these plots is presented in Table II. These values are in good agreement with *Ea* for the acid hydrolysis of acetanilide and some of the substituted acetanilides as reported by Karve and Kelkar (10). It is interesting to note that the energy of activation is very close to that reported for the acid hydrolysis of similar esters (14, 15), but less than that for the acid hydrolysis of substituted benzamides reported by Maloche and Laidler (9) which is around 23 Kcal. per mole. In the case of NAPAP which is a substituted anilide, it can be assumed that the N-phenyl substituent is sufficiently electrophilic to reduce the basicity of the amide nitrogen so that the response of NAPAP would be more like that of esters than amides as evidenced by the experimental data. Apparently the hydroxyl group in the *para* position in NAPAP

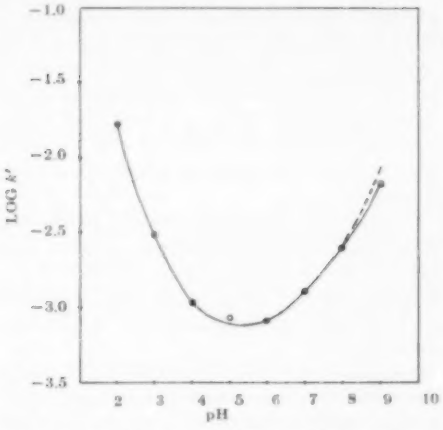


Fig. 9.—Log k', the specific reaction constant, against pH at 90°.

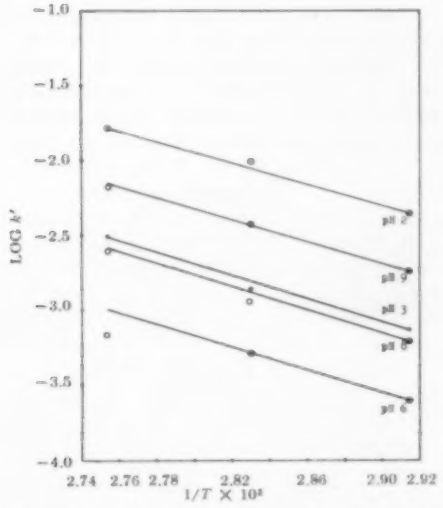


Fig. 10—A plot of log k' against $1/T$ showing the temperature dependence of the rate of hydrolysis of NAPAP at different pH's.

TABLE I.—SPECIFIC REACTION CONSTANT, k', FOR THE HYDROLYSIS OF NAPAP AT DIFFERENT pH'S AND AT DIFFERENT TEMPERATURES

pH	70°	80°	90°
	k' hours^{-1} × 10^4		
2	29.13	96.96	168.3
3	7.403	11.26	31.03
4	...	5.484	10.76
5	...	...	8.373
6	2.560	5.559	6.979
7	...	...	13.16
8	6.580	14.39	25.37
9	19.02	38.79	66.62

TABLE II.—ENERGY OF ACTIVATION, Ea, FOR THE HYDROLYSIS OF NAPAP AT DIFFERENT pH'S

pH	2	3	6	8	9
Ea, Kcal./ mole	16.69	17.99	17.42	17.99	17.42

does not have a significant effect on the energy of activation. This could probably be explained by the fact that the electron donor effect (+R) of the —OH group is offset by its inductive effect (−I) in the opposite direction.

The half lives for NAPAP in aqueous solutions at 25° were determined from the calculated specific reaction rate, k', for reactions at various pH's (Table III). A plot of log $t^1/_2$ against pH (Fig. 11) can be explained on the same basis as the plot of log k' *vs.* pH (Fig. 8) discussed earlier. As noted in these two figures, the hydrolysis of NAPAP is minimum in the pH range 5 to 7. The half lives of NAPAP at 25° in solutions of pH 5 and 6 are 19.8 and 21.8 years, respectively. Pharmaceutical formulations of NAPAP in liquid form therefore, would be extremely stable in this pH range.

TABLE III.—HALF LIFE PERIODS, $t^1/_2$, FOR THE HYDROLYSIS OF NAPAP AT DIFFERENT pH'S AND AT 25°

pH	$t^1/_2$, years	log $t^1/_2$
2	0.78	−0.1385
3	5.83	0.7653
4	15.39*	1.1872
5	19.78*	1.2963
6	21.80	1.3385
7	12.59*	1.1000
8	7.13	0.8529
9	2.28	0.3585

* Calculated using an average of the value for the activation energy at other pH's.

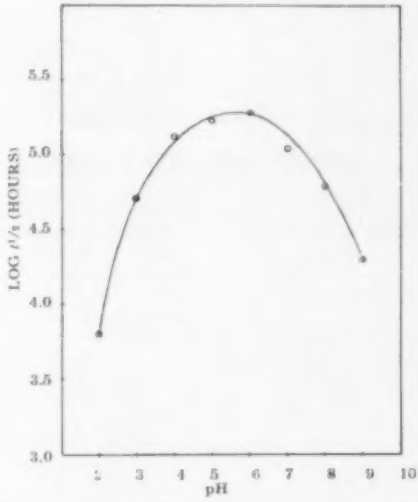
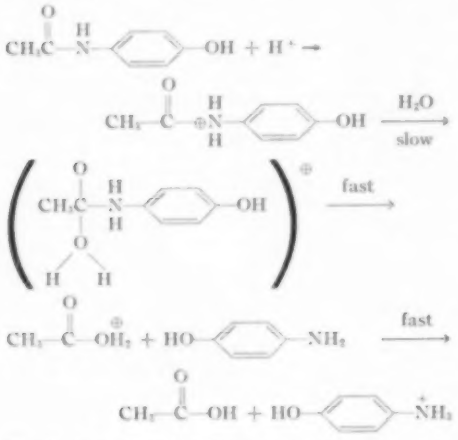
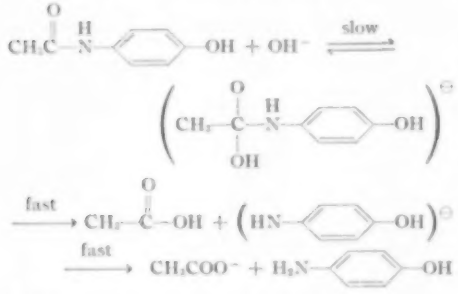


Fig. 11.—Log $t^1/_2$, the half-life period of the reaction, in hours, against pH at 25°.

The Mechanism of Hydrolysis.—From the data obtained, the mechanism of hydrolysis of NAPAP can be seen to follow the general pattern of hydrolysis of amides. In line with this, it seems likely that the acid hydrolysis takes the path



The basic hydrolysis follows the path



The rates of basic and acid hydrolysis are proportional, respectively, to (NAPAP)(OH^-) and (NAPAP)(H^+). The hydrolysis necessarily involves acyl nitrogen, rather than alkyl nitrogen, bond breakage. If this were not so, alcohols rather than amines would be formed in such hydrolysis.

SUMMARY AND CONCLUSION

1. The degradation of NAPAP in hydrochloric acid solutions of varying strengths at 70° and in buffer solutions of pH's ranging from 2–9 at 70, 80, and 90° have been studied. The degradation of NAPAP in aqueous solutions appear to be both an acid catalyzed and a base catalyzed reaction. It is first order with respect to the concentration of NAPAP and first order with respect to hydrogen and hydroxyl ion concentration.

2. The energy of activation, *Ea*, for the degradation of NAPAP in aqueous solution from pH 2 to 9 was found to be of the order of 17.5 Kcal./mole. This value is in good agreement with the value for *Ea* reported for similar compounds.

3. The half lives at 25° for NAPAP in aqueous solutions were determined from the calculated specific reaction constants for reactions at various pH's and at 25°. The half life for NAPAP in a buffered solution at pH 5 and 6 was calculated to be 19.8 and 21.8 years respectively. At pH 2 the half life is 0.73 years and at pH 9 the half life is 2.28 years, with intermediate values at intermediate pH's.

4. While formulating NAPAP in pharmaceuticals, it is desirable to keep the pH of the medium between 5 and 6 to obtain maximum shelf life for the product.

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Separation of Lipid-Shifting and Other Properties of Estrogens

By LAURETTA E. FOX, THOMAS D. MALEWITZ, and HOWARD E. MOSSBERG†

Rats maintained on high fat diets were treated with estrogens or their congeners. The actions of the compounds were highly specific. Total serum cholesterol was lowered 54.7 per cent by 1,1-dibenzyloxyphenyl-(2-*p*-anisyl)ethylene. This compound depressed testicular size 19.4 per cent but did not inhibit spermatogenesis appreciably. β-Diethylaminoethylfencholate hydrochloride lowered total serum cholesterol and depressed testicular size without depressing the process of spermatogenesis when measured by the criteria used in these experiments. There was no relation between the enlargement of the adrenals and the depression in weight of the testicles or the extent of depression of spermatogenesis.

ACCORDING to Engelberg (1) the best current concensus is that atherosclerosis is not an inevitable consequence of the wear and tear of aging but may be prevented or perhaps reversed. Since men show a significantly higher cholesterol level than premenopausal women (2) and arterial diseases predominate in males of comparable age (3), estrogens may prove useful in treating or preventing certain vascular syndromes. Estrogens alter the α/β lipoprotein and the cholesterol/phospholipid ratios (4). Patients with coronary diseases treated with estrogens show not only a lower cholesterol level but a lower recurrence of myocardial infarction than nontreated cardiac patients (5). The prolonged administration of available estrogens is limited by development of signs of gynecomastia, nausea, vomiting, lassitude, malaise, and diarrhea (6).

Hartley (7) prophesized that "in the future synthetic estrogens which have been tailored to accentuate specific biological manifestations for therapeutic advantage will be available." Malinow and Pellegrino (8) separated vascular from testicular effects to show that "the arterial protection afforded by estrogens is not mediated through an induced atropy of the testicle." Davies, *et al.* (9), found two compounds, possessing weak estrogenic actions, that shifted abnormal blood lipid levels toward average patterns. Cook, Edgrene, and Saunders (10) also selected several compounds with weak estrogenic actions that produced favorable lipid-shifting properties.

The purpose of this research was to seek a compound which would produce the beneficial shifting of blood lipids without producing undesirable side actions.

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MATERIAL AND METHODS

Compounds Used for Testing.—Alpha-estradiol, ethylene oxide polymer of estradiol as synthesized by Head and Lauter (11), chlorotrianisene, triphenylethylene, tri-*p*-anisylethylene, tri-*p*-methoxyphenylbromoethylene, 1,1-dibenzyloxyphenyl-2-(*p*-anisyl)ethylene, 9-(α-chloroanisal)9,10-dihydroanthracene, β-diethylaminoethylfencholate hydrochloride, diethylstilbestrol, hexestrol monomethyl ether, dienestrol, and 3,4-dianisylhexane, in pure crystalline form, were suspended uniformly or dissolved in sesame oil so that the doses indicated in Table I were contained in 0.05 ml. One series of doses was calculated to give approximately the same estrogenic activity and in the other series equal weights of each compound were administered.

Diets.—Diet I consisted of ground Purina chow; diet II of 93.5% ground Purina chow, 5.0% cholesterol, and 1.5% sodium cholate; and diet III of 53.5% ground Purina chow, 1.5% sodium cholate, 5.0% cholesterol, and 40.0% animal fat (lard, m. p. 40–42° C.).

Procedures with Animals.—Male albino rats of the Wistar strain weighing from 75 to 100 Gm. were randomly divided into 32 groups. Each group was housed in a separate community cage in an animal room maintained at 72 ± 2° F. The rats, treated with the above compounds, were offered one of three diets and water, *ad libitum*. The sesame oil, sesame oil suspension, or solution of each compound was injected intramuscularly twice weekly for two months.

Histological Techniques.—At the time interval indicated in Table I the rats were anesthetized with ether, exsanguinated from the abdominal aorta, and the heart, aorta, liver, adrenals, and testes were removed, dissected free of fat or fascia, and weighed to the nearest mg. Aliquots of these tissues were fixed in 5% formalin, dehydrated, and infiltrated according to the butyl paraffin mush method of Johnston, *et al.* (12). They were sectioned at 8 mμ and stained with Harris' hematoxylin and eosin as modified by Malewitz and Smith (13). Fresh frozen sections of heart, liver, and adrenal were tested for cholesterol by the Schultz method (14) and for lipid by Jackson's procedure (15).

Lipid Studies.—Total cholesterol was determined by the method of Abell, *et al.* (16), using a Spectronic 20 spectrophotometer at a wavelength of 600 mμ.

Serum phospholipids were extracted by the method of Zilversmith and Davis (17) and the phos-

phate determined by a modification of the method of Fiske and SubbaRow (18). The amount of lipid phosphorus was multiplied by a factor of 25 to give the amount of total phospholipid.

Total serum lipids were extracted by the method of Folch and Van Slyke (19) and determined colorimetrically by the method of Bragdon (20).

Total tissue cholesterol was determined on the frozen tissue aliquots of the adrenal glands, liver, and heart. Approximately 1 Gm. of tissue was transferred to a small mortar containing 8 Gm. of anhydrous sodium sulfate. The tissue was ground and dried ten minutes in an oven. After cooling it was further pulverized, transferred completely to a paper extraction shell, and placed in a Soxhlet apparatus. Redistilled chloroform, 25 ml., was poured over the ground tissue which was then extracted for ninety minutes. The extract was made to a volume of 25 ml. in a volumetric flask, an aliquot transferred to a dry test tube, and 2 ml. of freshly prepared acetic anhydride-sulfuric acid mixture (10 to 1 ratio by volume) was added. A standard was prepared by dissolving 0.8 mg. (or a smaller quantity if needed) of cholesterol U. S. P. in 10 ml. of chloroform. Chloroform was used as the blank. These standards were kept at 25°C. in a dark place for thirty minutes, then read on a spectrophotometer at 660 mμ.

Estrogenic Activity.—Compounds of unknown estrogenic potency were tested by the method of Allen and Doisy (21).

Testicular Depression.—The testes were graded for spermatic depression according to the following rating: 0 = no depression of sperm (Fig. 1); 1 = slight depression of sperm (Fig. 2); and 2 = almost complete depression of sperm (Fig. 3).

RESULTS AND DISCUSSION

Effects of the Various Diets.—The percentage of protein in each diet was: I, 25%; II, 21.56%; III, 13.38%. The energy/protein ratio (22) of diets I and II was 16.54 and of diet III, 43.50. The vitamin content of diet II was 5.5% less than that of diet I; and diet III, 53.5% of diet I. The fat content of diet I was 6%; of diet II, 10.5%; and of diet III, 48.2%. There were marked variations in food intake. Statistical analysis of the variations of food intake per week showed as much variation between groups as within the groups ($p = 0.01$), but at ($p = 0.1$) the food consumption correlated with increased body weight.

Body Weights and General Appearances.—The rats maintained on high fat diets (groups 3, 4, 6, and 7) gained less weight than those fed the low fat diet (groups 1, 2, and 5). The groups maintained on commercial rations (containing 6% fat and 25% protein) and treated with estrogens or their congeners, tended to gain less weight than their respective controls. In this respect rats differ from chickens (23, 24). When examined grossly, the rats that gained less weight showed a smaller volume of fat deposited in the subcutaneous layers of the skin and vice versa. Rats maintained on the high cholesterol diet and treated with estradiol and fencholate (groups 9 and 21) deposited more subcutaneous fat and gained more weight than their respective controls. Rats of groups 14, 23, 25, 27, 29, and 31 maintained on diet II gained less weight than the

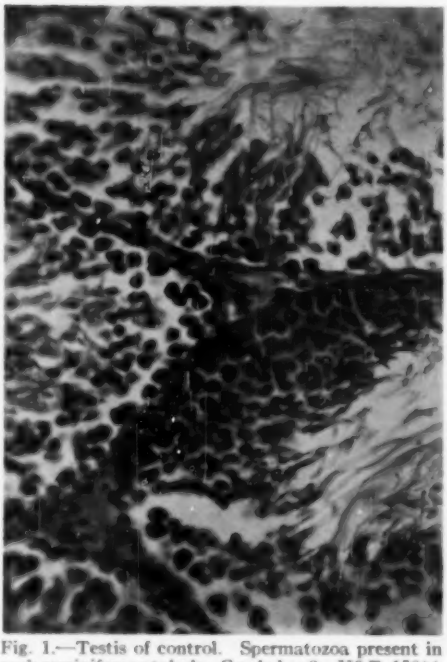

Fig. 1.—Testis of control. Spermatozoa present in each seminiferous tubule. Graded as 0. H&E. 150×

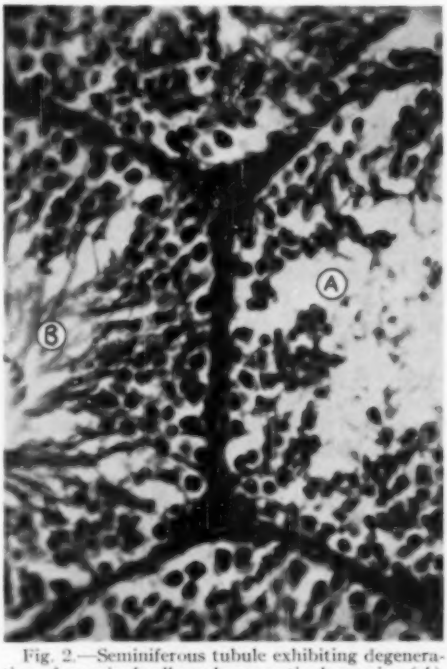

Fig. 2.—Seminiferous tubule exhibiting degeneration of germinal cells and spermatic depression (*A*). Normal seminiferous tubule (*B*). Graded as 1. H&E. 150 ×.

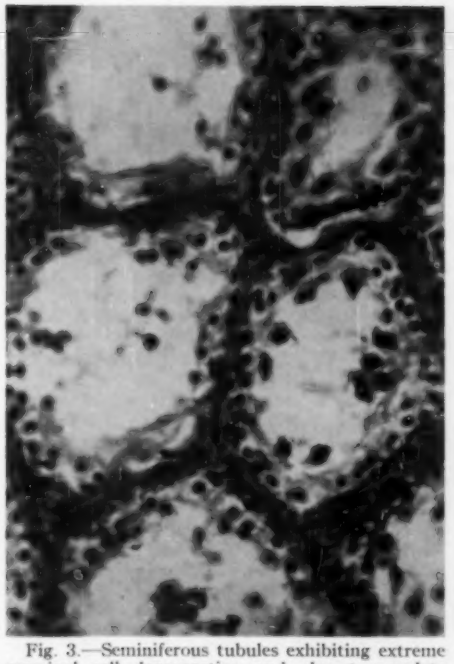

Fig. 3.—Seminiferous tubules exhibiting extreme germinal cell degeneration and almost complete spermatic depression. Graded as 2. H&E. 150×.

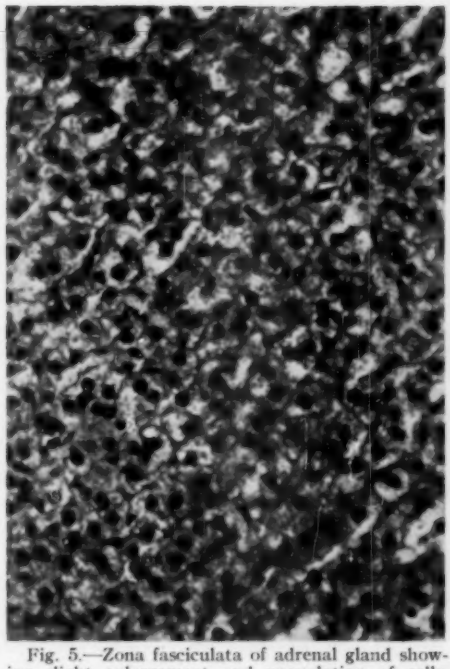

Fig. 5.—Zona fasciculata of adrenal gland showing slight enlargement and vacuolation of cells. H&E. 200×.

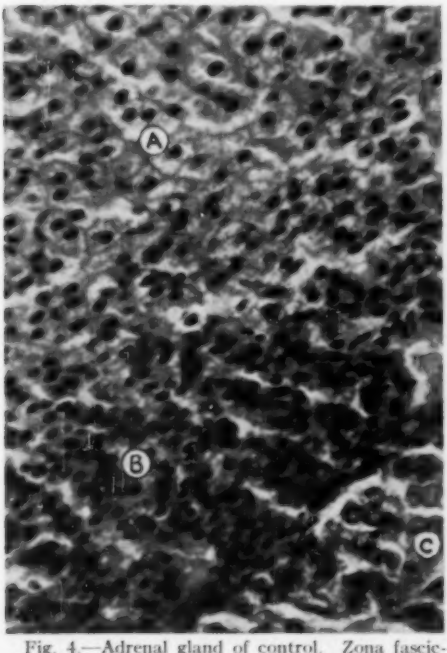

Fig. 4.—Adrenal gland of control. Zona fasciculata (*A*), zona reticularis (*B*), medullary region (*C*). H&E. 200×.

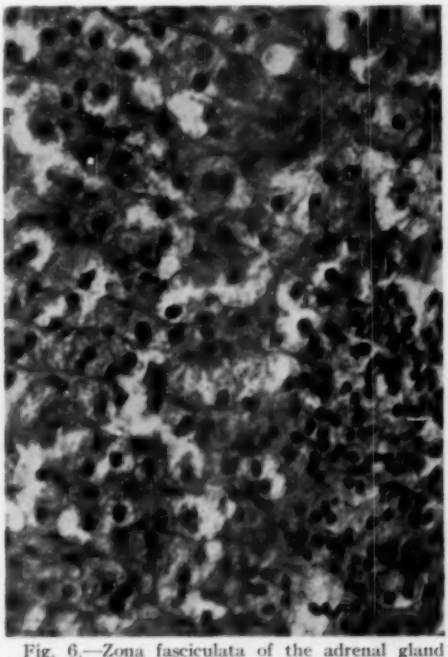

Fig. 6.—Zona fasciculata of the adrenal gland showing extreme enlargement and vacuolation of cells. H&E. 200×.

TABLE I.—BODY AND ORGAN WEIGHTS OF RATS MAINTAINED ON HIGH FAT DIETS AND TREATED WITH ESTROGENS OR CONGENERS

Group No.	Diet	No. of Animals	Drug	Dose, mg.	Estrogenic Potency (Ref.)	Final Body Weight Average Gm.	Total Cholesterol, mg./100 mg. Heart Tissue	Liver Weight, Gm./100 Gm. Liver Tissue	Total Cholesterol, mg./100 mg. Liver Tissue	Testicular Weight, Gm./100 Gm. Body Weight	Spermatic Depression	Adrenal Weight, mg./100 Gm. Body Weight	Total Cholesterol, mg./100 mg. Adrenal Tissue
1	I[a]	8	Control	....	....	92.0	159	5.10[b]	0.145	1.80	0	25[c]	3.96
2	I[d]	24	Sesame oil	....	....	220.6	...	5.03[b]	0.150	2.40	0	25[c]	...
3	II[d]	8	Sesame oil	....	....	269.3	...	5.45[b]	3.965	2.08	0	26[e]	...
4	III[d]	6	Sesame oil	....	....	276.7	...	4.41[b]	11.405	2.11	0	26[e]	...
5	I[a]	6	Sesame oil	....	....	284.3	160	6.00[b]	0.150	2.30	0	24[c]	3.96
6	II[a]	20	Sesame oil	....	....	276.7	158	6.58[f, g]	4.135	1.78	0	23[h]	6.50
7	III[a]	18	Sesame oil	....	....	275.7	160	6.58[m, n]	12.800	2.17	0	26[h]	6.40
8	I[a]	12	α-Estradiol U.S.P. XIV	0.025	10% as active as estrone (41)	265.0	160	4.24[b]	0.500	1.45	1	31[c, g]	2.04
9	II[a]	12	α-Estradiol U.S.P. XIV	0.025	10% as active as estrone	321.7	161	5.38[g, i]	7.380	1.37	1	27[h]	7.10
10	III[a]	6	α-Estradiol U.S.P. XIV	0.025	10% as active as estrone	242.5	162	7.22[f, g]	13.300	1.44	1	22[h]	8.18
11	III[a]	6	Polymer of estradiol	0.250	40 mg.[j]	270.0	159	7.17[f, n]	17.400	1.70	1−	32[h]	8.75
12	I[a]	6	Chlorotrianisene	62.50	25γ[l] (26)	260.0	159	4.25[i]	0.660	0.76	2+	25[h]	1.90
13	I[a]	6	Chlorotrianisene	62.50	25γ[l]	245.0	162	4.39[i]	0.660	0.78	2+	33[h]	1.93
14	II[a]	12	Chlorotrianisene	62.50	25γ[l]	179.0	160	7.21[g, m]	9.560	0.91	2+	34[h]	4.85
15	III[a]	6	Chlorotrianisene	62.50	25γ[l]	159.0	160	9.71[g, n]	15.250	0.70	2+	38[h]	5.91
16	III[a]	6	Triphenylethylene	0.250	10 mg.[o] (42)	293.0	160	7.26[f, g]	12.400	1.66	1−	13[h]	12.00
17	III[a]	6	Tri-p-anisylethylene	0.250	12,000[p] (43)	299.2	160	6.61[f, g]	14.500	1.47	1−	22[h]	9.90
18	III[a]	6	Tri-p-methoxyphenylbromoethylene	0.250	50γ[l] (43)	216.0	160	7.72[f, g]	12.000	1.15	1	38[h]	6.20
19	III[a]	6	1,1-Dibenzyloxyphenyl-2-(p-anisyl)ethylene	0.250	500γ[p] (43)	251.0	161	6.94[f, g]	16.480	1.75	1−	23[h]	5.08
20	III[a]	6	9-(α-Chloroanisal)-9,10-dihydroanthracene	0.250	2000γ[p] (43)	279.2	160	7.03[f, g]	14.440	1.55	0	24[h]	8.00
21	II[a]	8	β-Diethylaminoethylfencholate hydrochloride	0.250	120 mg.[p]	320.0	161	4.11[f, g]	8.250	0.88	0	24[h]	4.65
22	I[a]	6	Diethylstilbestrol	0.250	1γ[l] (42)	227.0	160	4.66[i]	0.150	0.67	2	19[h]	2.75
23	II[a]	20	Diethylstilbestrol	0.250	1γ[l]	265.8	160	6.68[f, g]	10.000	0.67	1+	33[h]	8.70
24	III[a]	6	Diethylstilbestrol	0.250	1γ[l]	236.7	160	5.93[f, n]	8.430	0.81	1+	31[h]	6.50
25	II[a]	8	Hexestrol monomethyl ether	0.750	2.5γ[l] (44)	262.5	162	5.35[m, n]	8.430	0.47	2+	36[h]	7.48
26	I[a]	6	Dienestrol	0.500	1.5γ[l] (42)	212.0	159	5.08[i]	0.710	0.65	2+	69[h]	3.25
27	II[a]	6	Dienestrol	0.500	1.5γ[l]	218.0	159	7.50[g, m]	17.240	0.52	2+	32[h]	5.04
28	III[a]	12	Dienestrol	0.50	1.5γ[l]	173.0	159	11.14[g, m]	17.500	0.49	2+	44[h]	8.15
29	II[a]	6	3,4-Dianisylhexane	0.250	50.0γ[l] (43)	226.4	159	4.67[g, m]	6.001	0.73	2+	33[h]	5.50
30	I[a]	12	3,4-Dianisylhexane	0.750	50.0γ[l] (43)	228.0	159	5.09[g, m]	2.040	0.67	2+	33[h]	2.04
31	II[a]	6	3,4-Dianisylhexane	0.750	50.0γ[l]	230.0	162	6.54[m, n]	6.580	0.85	2+	47[h]	5.50
32	III[a]	6	3,4-Dianisylhexane	0.750	50.0γ[l]	206.0	162	7.55[m, n]	12.150	0.68	2+	23[h]	3.50

[a] Fed diet zero days. [b] Liver normal (see Fig. 7). [c] Adrenal cells normal (see Fig. 4). [d] Fed diet thirty days. [e] Fed diet sixty days. [f] Liver cells enlarged and vacuolated (see Fig. 8). [g] Few areas of degeneration. [h] Cells of zona fasciculata enlarged and vacuolated (see Fig. 5). [i] Liver almost normal, condition between Figs. 7 and 8. [j] Active in rat. [k] Cells of zona fasciculata greatly enlarged and highly vacuolated (see Fig. 6). [l] Equivalent to one rat unit. [m] Liver cells greatly enlarged with extreme vacuolation (see Fig. 9). [n] Prominent areas of degeneration. [o] Gives 100% activity in ovariectomized rat. [p] Inactive in rat.

controls. Rats fed the high fat-cholesterol diet and treated with triphenylethylene and tri-p-anisylethylene (groups 16, 17, and 20) gained more weight than their respective controls. Animals of groups 10, 11, 15, 18, 19, 24, 28, and 32 gained less weight than their respective controls.

The rats treated with estrogens showed more roughness of coat than their respective controls. Of the controls, those on the high fat diet showed more tendency to roughness of coat.

Fifty per cent of the animals in groups 8, 9, and 10 developed a patchy alopecia during the third week of treatment similar to that described by Ingle and Baker (25). This condition was not evident in group 11 and was less marked in animals receiving other estrogens than estradiol.

Histological Examination of the Adrenals.—The normal histological characteristics of the adrenal are shown in Fig. 4. Cells of the zona fasciculata of rats maintained on high fat diets showed some enlargement and vacuolation (Table I). The amount of enlargement and vacuolation was graded into two degrees as shown in Figs. 5 and 6. The amount of enlargement and vacuolation of the cells of the zona fasciculata is characteristic of the diet and the compound used for treatment.

Frozen sections of adrenals stained for cholesterol and lipid correlated with the adrenal tissue cholesterol levels are recorded in Table I. Cholesterol and fats were concentrated only in the zona fasciculata.

Weight and Size of Adrenals.—The adrenals of the rats maintained on the high cholesterol diet increased in size and weight. This condition was more marked in these groups of animals fed diet III. The increase in the weight of the adrenals also varied with the estrogen used for treatment. The greatest increase in weight of the adrenals was in group 26 maintained on diet I and treated with dienestrol, as reported previously by Gertler, *et al.* (26), and in group 31 receiving dianisylhexane. While the rats treated with the smaller dose of chlorotrianisene (group 12) did not show an appreciable increase in weight of the adrenals, as reported by Thompson and Werner (27), those treated with larger doses of this drug did show some enlargement (groups 13, 14, and 15).

According to Bernstein (28) the adrenal glands of male rats increased in weight only after the administration of estrogens, and Brand (29) found that as long as the pituitary was present the administration of estrogens always led to an increase in adrenal weight. Long (30) commented that "The enlargement of the adrenal cortex . . is dependent on stimulation of the anterior pituitary."

Histological Examination of Livers.—Size and weight of the liver increased after feeding a high cholesterol diet and this condition became more marked after adding fat to the cholesterol diet. Livers which increased in weight were characterized by enlarged and vacuolated hepatic cells. These cells increased in cholesterol content as determined by the Schultz test, and in lipid content as determined by the Jackson procedure. Areas of degeneration were observed after treatment with certain drugs (Table I). The severity of the vacuolation of the cells and the amount of degeneration observed is tabulated for each group in Table I. The severity of cellular enlargement and vacuolation is shown in Figs. 7, 8, and 9.

Laffont and LeCannelier (31) reviewed the variable effects of estrogens on the liver. Some workers observed vacuolation; others reported fatty degeneration.

Histological Examination of Hearts and Aortae.—No increases in the concentration of cholesterol in the myocardia were observed, although Stamler and co-workers (24) observed increases in total cholesterol of the myocardia of chickens on atherogenic diets. Frozen sections of heart were negative for cholesterol and lipid as determined by the Schultz cholesterol test and the Jackson lipid procedure.

Although the coronaries and aortae of the rats maintained on the high fat diets and treated with estrogens, the principals, did have some congestion in their vessels, no areas of fatty infiltration, foam cells, thickened intimas, or atheromas were observed, although other workers have observed lesions in the coronaries or aortae of rats (8, 32, 33).

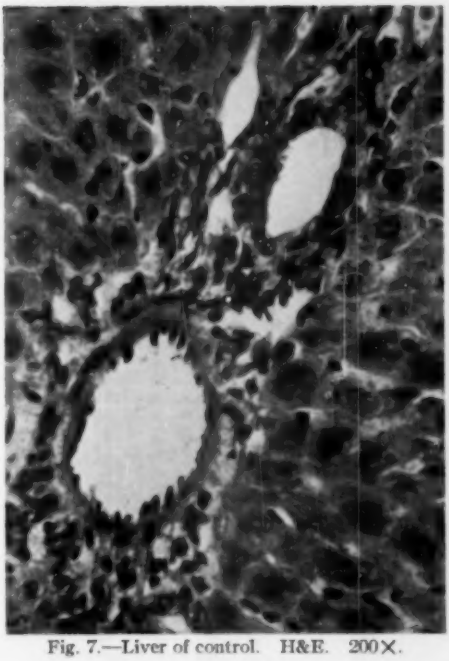

Fig. 7.—Liver of control. H&E. 200×.

Arterial lesions have been produced in rats by feeding a high lipid cholesterol diet with a nutritional deficiency of methionine (32) or high fat-cholesterol diets alone (34). Coronary thrombosis and myocardial infarction were induced by feeding a diet high in cholesterol and butter fat while administering thiouracil (33).

Serum Lipid Levels.—High cholesterol and high fat-cholesterol diets produced an increase in the total serum cholesterol, phospholipids, and neutral fats, as shown in Table II. The experiments reported by Fellios (35) indicated that "estradiol favored an increased cholesterolemia among male rats." This was confirmed by the work of Moskowitz and co-workers (36), or Malinow and Pellegrino (8).

The difference in cholesterolemic responses between these experiments and those of other workers cannot be attributed to the inhibition of cholesterol synthesis by cholic acid (37), since the diets used in both these experiments and those of Fellios (35) contained 1.5% sodium cholate, but those used by other workers (8, 36) did not.

The fall in total cholesterol levels after the administration of estrogens in these experiments (Table II) showed that the decrease in cholesterol level obtained was similar to the response reported in chickens by Stamler and co-workers (4). The rat has a lower concentration of high density lipoprotein components (those above S_f 30) than other species, as reported by Lewis (38). The C/P ratio is determined more by the relative amount of high density lipoproteins compared to the low density

TABLE II.—LIPOID CONCENTRATION IN SERUM

Group No.	Total Serum Cholesterol, mg. %	Phospholipid, mg. %	Neutral Fat, mg. %	C/P Ratio
1	70.12 ± 5.60	90.10	190.61	0.78
2	72.30 ± 5.19	95.25	194.63	0.76
3	149.60 ± 34.60	100.00	492.67	1.50
4	191.70 ± 14.18	95.25	746.90	2.01
5	71.12 ± 6.20	96.01	196.51	0.74
6	205.60 ± 26.97	133.33	705.58	1.54
7	246.90 ± 71.28	166.00	614.29	1.49
8	32.35 ± 11.00	120.00	477.61	0.27
9	88.00 ± 24.20	141.00	666.54	0.62
10	111.70 ± 18.54	158.00	520.74	0.71
11	269.30 ± 94.75	166.00	688.14	1.62
12	65.01 ± 7.80	91.05	410.62	0.71
13	25.90 ± 6.90	91.00	412.31	0.29
14	134.00 ± 39.00	133.00	626.36	1.00
15	103.50 ± 30.70	141.00	457.82	0.74
16	238.60 ± 63.43	141.00	354.03	1.69
17	224.50 ± 69.42	130.00	508.48	1.73
18	210.30 ± 70.84	150.00	506.76	1.40
19	111.90 ± 28.85	150.00	501.46	0.75
20	237.80 ± 86.90	146.00	440.95	1.63
21	65.00 ± 15.43	83.25	487.72	0.78
22	25.74 ± 4.94	125.00	383.29	2.06
23	105.50 ± 44.79	146.00	521.03	0.72
24	122.20 ± 34.56	133.00	519.48	0.92
25	40.00 ± 7.20	120.75	587.53	0.33
26	20.10 ± 4.10	91.00	332.18	0.22
27	146.60 ± 28.70	113.00	348.52	1.30
28	137.90 ± 44.00	150.00	320.65	0.92
29	65.32 ± 13.50	123.00	449.10	0.53
30	21.72 ± 5.88	141.00	325.42	1.54
31	58.30 ± 17.16	125.00	708.06	0.47
32	35.00 ± 6.25	125.00	718.90	0.28

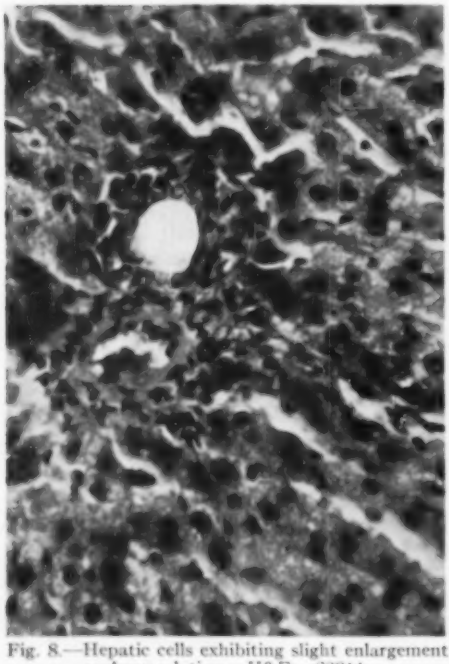

Fig. 8.—Hepatic cells exhibiting slight enlargement and vacuolation. H&E. 200×.

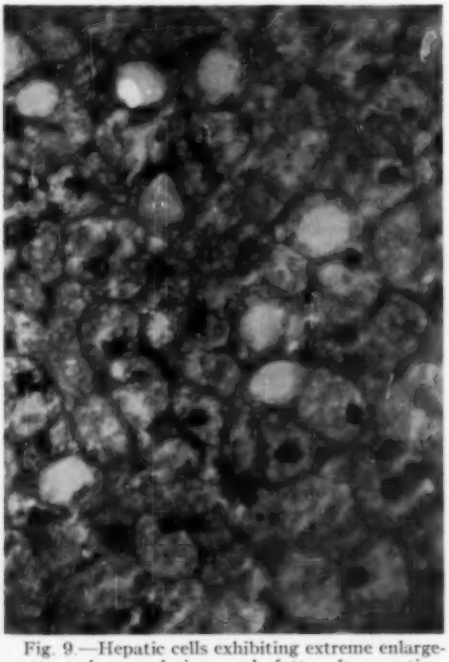

Fig. 9.—Hepatic cells exhibiting extreme enlargement and vacuolation and fatty degeneration. H&E. 200×.

lipoproteins than by the distribution of cholesterol among the groups from 3 to 100 S_f (4). In the high density lipoproteins the weight ratio of cholesterol to phospholipid is about one-half, while molecules of the S_f 10 to 20 class may have a ratio as high as 1.3 (4). Therefore C/P ratios of rats cannot be expected to be as significant as those of other species. The C/P ratios obtained in these experiments were higher values than other workers (8) reported.

Statistical analysis of the total cholesterol of the sera of the principals showed that at $p = 0.01$ only groups 11, 16, 17, 18, and 20 receiving the polymer of estradiol, triphenylethylene, tri-*p*-anisylethylene, tri-*p*-methoxyphenylbromethylene, 9-(α-chloranisal) 9,10-dihydroanthracene, respectively, showed insignificant lowering of the total cholesterol.

In all animals maintained on diet I, treatment with estrogen increased the neutral fat of the serum. All principals, maintained on diet II, showed a lower neutral fat level than the controls. Except for group 32, the neutral fat of the serum was lower in rats maintained on diet III than II.

As Long (30) comments, the actions of estrogens on metabolism were probably mediated through the hypophysis. The metabolic effects were specific for each compound studied (Tables I and II).

Estrogenic Activity.—The epioxide polymer of α-estradiol did not produce estrus in the ovariectomized rat when administered in lower dosage than 40 mg., in divided doses, at two-day intervals. β-Diethylaminoethyl fencholate hydrochloride was inactive as an estrogen when administered in two doses of 60.0 mg. each, according to the Allen-Doisy assay (21). The estrogenic activity of all other compounds used in this study was not determined experimentally. The values used and the reference from which these values were obtained were recorded in Table I.

Depression of Testicular Size and Weight.—Compounds which lowered cholesterol levels depressed testicular size and weight. There was less depression of testicular size for each per cent lowering of total cholesterol of the serum with Group 19, treated with 1,1-dibenzyloxyphenyl-(2-*p*-anisylethylene), as is shown in Fig. 10. Bacon and Kirkman (39) suggested that estrogens may act to some degree directly on the gonads rather than by inhibition mediated through the hypophysis.

Depression of Spermatogenesis.—Spermatic depression was characteristic of all animals treated with these compounds except groups 20 and 21. The dihydroanthracene derivative did not lower total cholesterol (Table II). The fencholate (group 21) decreased total cholesterol. Although the testicles of those two groups of animals weighed less than those of the controls, sperm were present and little spermatic depression was recorded by the criteria used for grading spermatic depression. Depression of testicular size or weight may be produced by a compound without depression of spermatogenesis.

Relation of Adrenal Increase in Weight to Testicular Depression.—Increase in weight of the adrenals did not protect the testicle against depression in weight or increase in weight of the adrenal did not correlate with degree of depression of spermatogenesis. It was hypothesized by Lynch (40) that an enlarged adrenal would produce more androgens which might protect the testicles.

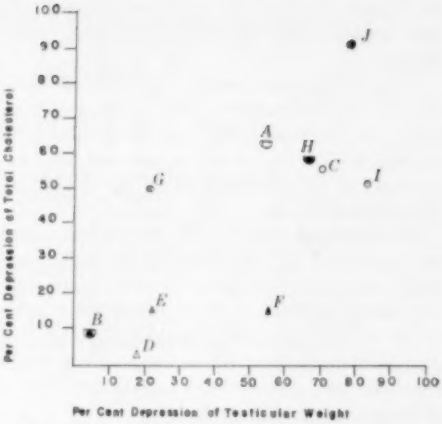

Fig. 10.—Relation of depression of total serum cholesterol to per cent depression of testicular weight. Diet III. A, α-estradiol; B, polymer of estradiol; C, chlorotrianisene; D, triphenylethylene; E, tri-*p*-anisylethylene; F, tri-*p*-methoxyphenylethylene; G, 1,1-dibenzyloxyphenyl-2-(*p*-anisyl)ethylene; H, diethylstilbestrol; I, dienestrol; and J, 3,4-dianisylhexane.

SUMMARY AND CONCLUSIONS

Rats fed high cholesterol and high fat-cholesterol diets tended to produce a roughness of coat and their body weights increased. Total serum cholesterol, phospholipid, and neutral fat levels of the serum increased. The livers of these animals increased in weight. The hepatic cells increased in size, became vacuolated, and stored more cholesterol and lipids. The adrenals tended to increase in weight and size. No effects were observed on testicular size or spermatogenesis. There was no detectable increase in cholesterol levels of the myocardium. No lesions were observed in the coronaries or aortae.

The total serum cholesterol of the rats treated with α-estradiol was decreased. Cholesterol concentration in the liver was increased but the concentration in the myocardium was not affected. Testicular weight was depressed and spermatogenesis was inhibited.

The epioxide polymer of estradiol was devoid of activity to depress serum cholesterol. It depressed spermatogenesis and testicular size less than estradiol.

Chlorotrianisene lowered total cholesterol levels but also depressed testicular weight and spermatogenesis.

1,1 - Dibenzyloxyphenyl - 2 - (*p* - anisyl)-ethylene, in which the two anisyl groups of the chlorotrianisene were substituted with benzyloxyphenyl groups, produced only slight reduction in weight in the testicle (19.4 per cent)

but markedly lowered serum cholesterol. It also caused only slight depression of spermatogenesis.

Triphenylethylene which possesses approximately one-thousandth the estrogenic activity of chlorotrianisene has little action in shifting lipid levels of the serum and also has little action on spermatogenesis, but it did depress testicular size.

Substitution of the chloride of chlorotrianisene with hydrogen or bromide produced compounds which decreased estrogenic action and also decreased the activity in shifting cholesterol levels of the serum. Trimethoxyphenylbromoethylene depressed testicular weight more than tri-*p*-anisylethylene, although both had the same action in depressing spermatogenesis.

β-Diethylaminoethylfencholate hydrochloride did not possess estrogenic action under these conditions of experimentation. It did not appreciably affect the adrenals. Although it depressed testicular weight 50.6 per cent below the control, it did not affect spermatogenesis as measured by the criteria outlined in these methods. It did lower total serum cholesterol 68.3 per cent.

Diethylstilbestrol and the related compounds tested lowered total serum cholesterol and depressed testicular weight and spermatogenesis.

No relationship was observed between the increase in weight of the adrenals and the protection of the testicle.

In no case was the concentration of cholesterol in the myocardium increased. No coronary or arterial lesions were observed.

Feeding high fat-cholesterol diets caused the hepatic cells to enlarge and become engorged with fats or cholesterol. The degree of degeneration of the liver cells was associated with the action of the estrogen.

The cholesterolemic response of the rats used in these experiments was similar to that produced in chickens, but neither phospholipid levels nor C/P ratios proved of value in evaluating the estrogens.

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Studies on Quinoline Derivatives as Anti-infective Agents I

By S. M. BAHAL, M. R. BAICHWAL, and M. L. KHORANA

A systematic study of 120 derivatives of quinoline for their bacteriostatic activity against *Micrococcus pyogenes* var. *aureus* and *Salmonella typhosa* and for fungistatic activity against *Aspergillus niger* has been reported. Thirteen compounds selected from the preliminary screening were further examined for their activity against *Micrococcus pyogenes* var. *aureus*, *M. pyogenes* var. *albus*, *Streptococcus β-hemolyticus*, *Salmonella typhosa*, *Salmonella paratyphi A*, *S. paratyphi B*, *Shigella dysenteriae*, and *Escherichia coli*. 5-Acetyl-, 5-nitro-, 7-bromo-5-nitro-, 5-formyl-, and 7-bromo-5-formyl- 8-hydroxyquinolines were found to be bacteriostatic at 1 to 5 mcg./cc. against most of the Gram-positive as well as the Gram-negative organisms. High activity of 8-acetoxyquinoline, without a free hydroxyl group and hence nonchelating, is noteworthy. However, the stability of this compound will have to be investigated further.

THE EMERGENCE of promising compounds from the studies previously reported from this laboratory (1–3) on the relationship of the antibacterial and antifungal properties with the chemical constitution of α- and β-naphthol derivatives prompted similar studies in the quinoline series. A few compounds such as, 5,7-dichloro-2-methyl-, 5,7-diiodo-, and 5-chloro-7-iodo-8-hydroxyquinoline have found common use as anti-infective agents from the work already reported in the literature. Hence, it was considered that a more extensive and systematic study of quinoline derivatives might not be potentially unfruitful. In the field of activity, particularly against the dermatophytic fungi, the only systematic investigations have been those of Mason (4), Oster and Golden (5), and Woodcock and Rogers (6).

In the present work, 120 derivatives of quinoline and 8-hydroxyquinoline have been studied *in vitro* for their antibacterial and antifungal activity. The compounds which were found to possess reasonably high activity have been further studied for their antibacterial spectra.

In this report, however, derivatives such as styrylquinolines and quinolyl-acrylic acids, chalcones of 5-acetyl-8-hydroxyquinoline with various substituted benzaldehydes, and the anilinobenzyl derivatives which showed little activity against either bacteria or fungi, have not been included.

EXPERIMENTAL

Synthesis of Compounds.—Most of the compounds were prepared and purified by methods already described in literature; hence, these are not reported separately.

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The authors wish to express their gratitude to the Bombay State Industrial Research Committee for the financial grant and a fellowship to one of us (SMB) during the tenure of which the major part of this work was completed.

Antibacterial Activity.—Screening for bacteriostatic activity was made using *Micrococcus pyogenes* var. *aureus* (FDA 209) and *Salmonella typhosa* (Lister), and following the serial broth-dilution method of Mckee, Rake, and Menzel (7). Various concentrations of the test substance were obtained by the addition of an appropriate quantity of an alcoholic stock solution to 5 cc. double strength nutrient broth. In no case did the quantity of the alcoholic (95%) solution added exceed 0.1 cc. Similar quantity of alcohol alone in 5 cc. broth did not cause bacteriostasis. The results were observed after incubation at 37° for twenty-four hours and further confirmed after forty-eight hours. The minimum inhibition concentrations in mcg./cc. are recorded in Table I.

For the determination of antibacterial spectra of the more active compounds, *Micrococcus pyogenes* var. *aureus*, *M. pyogenes* var. *albus*, *Streptococcus hemolyticus*, *Salmonella typhosa*, *Salmonella paratyphi* A, *Salmonella paratyphi* B, *Shigella dysenteriae*, and *Escherichia coli* were used with double strength broth containing 20, 10, 5, 2, and 1 mcg./cc. of the test substance. Otherwise, the method was essentially the same as that followed for preliminary screening. The results are recorded in Table II.

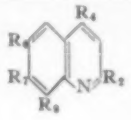
Antifungal Activity.—The agar-streak and the agar cup-plate methods were followed for the fungistatic screening. The former test procedure gave the minimum inhibition concentration values and the latter, an idea of the power of diffusion as well as the dosage-response curves.

Agar-Streak Method.—Schamberg and Kolmer's procedure (8) was followed for the purpose. The solutions of the compounds were prepared in alcohol (95%) and dilutions made in Sabouraud's agar to obtain the concentrations of 200, 100, 50, and 10 mcg./cc. Results were noted after incubation at 25–27° for five days. The minimum inhibition concentrations in mcg./cc. are recorded in Table I.

Agar Cup-Plate Method.—A modification (9) of the technique of Burlingame and Reddish (10) was adopted. Diameters of zones of complete inhibition, with 0.25% w/v alcohol (95%) solutions of the compounds, obtained after incubation at 25–27° for forty-eight hours, are recorded in Table I. Alcohol (95%) alone gave no zone of inhibition.

The compounds found promising were further studied by the same procedure using lower concen-

TABLE I.—BACTERIOSTATIC AND FUNGISTATIC ACTIVITIES OF QUINOLINE DERIVATIVES



Serial No.	Position of Substitution					Activity Against			
	R_2	R_4	R_6	R_7	R_8	*M. pyogenes* var. *aureus*.[a]	*S. typhosa*[a]	*A. niger* [c]	*A. niger* [b]
1	H	H	H	H	H	e	e	d	..
2	H	H	OH	H	H	25	50	d	..
3	CH_3	H	H	H	H	e	e	d	..
4	H	CH_3	H	H	H	e	e	d	..
5	H	H	CH_3	H	H	e	e	d	..
6	H	H	H	CH_3	H	e	e	d	..
7	H	H	H	H	CH_3	e	e	d	..
8	CH_3	CH_3	H	H	H	e	e	d	..
9	CH_3	H	CH_3	H	H	e	e	d	..
10	CH_3	H	H	CH_3	H	e	e	d	..
11	CH_3	H	H	H	CH_3	e	e	d	..
12	CH_3	H	Cl	H	H	e	e	200	21
13	CH_3	H	H	H	Cl	e	e	200	12
14	CH_3	H	NO_2	H	H	e	e	d	..
15	CH_3	H	H	H	NO_2	e	e	d	..
16	CH_3	OH	H	H	H	35	50	d	..
17	CH_3	H	OH	H	H	35	50	d	..
18	OH	CH_3	H	H	H	50	100	d	..
19	CH_3	OH	CH_3	H	H	100	100	d	..
20	CH_3	OH	Cl	H	H	50	100	d	..
21	CH_3	OH	H	H	Cl	50	100	d	..
22	H	H	CH_2Ph	H	H	e	e	d	..
23	H	H	OCH_3	H	H	e	e	d	..
24	H	H	OC_2H_5	H	H	e	e	d	..
25	H	H	NO_2	H	H	e	e	d	11
26	H	H	H	NO_2	H	e	e	d	..
27	H	H	H	H	NO_2	e	e	200	12
28	H	H	NH_2	H	H	e	e	d	..
29	H	H	H	NH_2	H	e	e	d	..
30	H	H	H	H	NH_2	e	e	d	..
31	H	H	SO_3H	H	H	e	e	d	..
32	H	H	H	SO_3H	H	e	e	d	..
33	H	H	H	H	SO_3H	e	e	d	..
34	H	H	Cl	H	H	e	e	d	13
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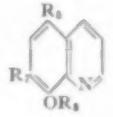
36	H	H	H	H	Cl	e	e	d	..



Serial No.	Position of Substitution			Activity Against			
	R_2	R_7	R_8	*M. pyogenes* var. *aureus*.[a]	*S. typhosa*[a]	*A. niger* [c]	*A. niger* [b]
37	H	H	H	10	50	10	Clear
38	CH_3[e]	H	H	50	50	50	43
39	C_2H_5	H	H	10	e	50	49
40	H	H	$COCH_3$	5	20	10	75
41	H	H	COC_6H_5	e	e	50	42
42	H	H	CH_3	e	e	100	13
43	H	H	C_6H_5	5	50	50	38
44	Br	Br	CH_3	100	100	100	18
45	CH_3	H	CH_3	e	e	100	15
46	$COCH_3$	H	H	2	20	50	57
47	$COCH_3$	H	COC_6H_5	20	e	50	44
48	Cl	H	H	20	50	50	..
49	Br	H	H	50	50	50	14
50	I	H	H	50	100	50	14
51	Cl	Cl	H	20	50	50	24
52	Br	Br	H	50	50	50	16
53	I	I	H	100	100	100	12
54	Cl	I	H	10	20	100	21
55	Cl, CH_3[e]	Cl	H	0.2	50	10	24

TABLE I.—(*Continued*)

56	SO_3H	H	H	[c]	[c]	[d]	..
57	SO_3H	I	H	[c]	[c]	[d]	..
58	NO	H	H	50	50	100	19
59	NO_2	H	H	1	5	10	59
60	NO_2	NO_2	H	[c]	[c]	200	15
61	NO_2	Br	H	2	5	10	45
62	NO_2	I	H	5	10	100	22
63	NH_2	H	H	100	100	200	26
64	NHAc	H	H	100	[c]	200	15
65	CHO	H	H	2	5	50	66
66	CHO	Br	H	2	5	50	53
67	CHO	NO_2	H	100	[c]	[d]	14
68	$COCH_3$	NO	H	20	100	100	41
69	COC_6H_5	H	H	2	50	50	27
70	$CH_2C_6H_5$	H	H	50	[c]	10	19
71	COOH	H	H	[c]	[c]	[d]	..
72	CONH-Ph	H	H	10	[c]	50	..

[a] M.I.C. in mcg./cc. [b] Average diameter of zone of inhibition in mm. with 0.25% w/v solutions. [c] M.I.C. greater than 100 mcg./cc. [d] M.I.C. greater than 200 mcg./cc. [e] Substituent in position 2.

TABLE II.—ANTIBACTERIAL SPECTRA OF SELECTED 8-HYDROXYQUINOLINE DERIVATIVES[a]

No.[b]	Derivative of 8-Hydroxyquinoline	*M. pyogenes* var. *aureus*	var. *albus*	*S. hemolyticus*	*S. typhosa*	*S. paratyphi* A	B	*S. dysenteriae*	*E. coli*
37	8-Hydroxyquinoline	10	5	10	50	50	50	20	50
38	2-Methyl-8-hydroxyquinoline	50	50	50	50	50	50	50[c]	50
39	5-Ethyl-8-hydroxyquinoline	10	10	10	100[c]	100[c]	100[c]	50	100[c]
40	8-Acetoxyquinoline	5	5	10	20	20	10	20	50
46	5-Acetyl-8-hydroxyquinoline	2	5	10	20	10	10	10	20
54	5-Chloro-7-iodo-8-hydroxyquinoline	10	10	10	20	50	20	10	50
55	5,7-Dichloro-2-methyl-8-hydroxyquinoline	0.2	0.5	2	50	50	50	20[c]	50[c]
59	5-Nitro-8-hydroxyquinoline	1	5	5	5	5	5	5	10
61	7-Bromo-5-nitro-8-hydroxyquinoline	2	2	20	5	5	5	20	20
62	7-Iodo-5-nitro-8-hydroxyquinoline	5	2	10	10	20	5	20	50[c]
65	5-Formyl-8-hydroxyquinoline	2	2	5	5	5	5	10	20
66	7-Bromo-5-formyl-8-hydroxyquinoline	2	5	20	5	20	10	20	50[c]
69	5-Benzoyl-8-hydroxyquinoline	2	5	20	50	50	20	50[c]	50[c]

Cultures of the organisms were obtained from the Haffkine Institute, Bombay, India.
[a] Minimum inhibition concentration in mcg./cc. [b] Numbers refer to compounds listed in Table I. [c] Greater than the mcg./cc. indicated.

trations, *viz.*, 0.25, 0.1, 0.05, 0.025, and 0.0125% w/v. The dosage-response curves obtained by plotting the average zone diameters of complete inhibition against log of concentrations of ten selected compounds are given in Fig. 1.

DISCUSSION

The parent compound quinoline is inactive against *M. pyogenes* var. *aureus*, *S. typhosa*, and *A. niger*. Introduction of a chloro, nitro, amino, alkyl, or sulfonic acid group does not impart any activity to the basic compound, and the result of introducing a chloro or a nitro group in quinaldine is the same. However, introduction of a hydroxyl group gives high bacteriostatic as well as fungistatic activity to the quinoline nucleus. Introduction of a hydroxyl group in quinaldine also imparts some activity. 8-Hydroxyquinoline has already been reported to be the most active of all the seven isomers of 8-hydroxyquinoline (11, 12); hence, further work was limited mostly to study of derivatives of 8-hydroxyquinoline.

Considering the effect of substitution in 8-hydroxyquinoline, it is found that while a methyl group in position 2 (No. 38) does not enhance the bacteriostatic activity, an ethyl group in position 5 (No. 39) enhances the activity only against *M. pyogenes* var. *aureus*. Introduction of a chloro or a bromo group is more promising than an iodo group (Nos. 48–55). Introduction of sulfonic acid group greatly curtails the activity (Nos. 56, 57). While the methyl ether of 8-hydroxyquinoline and its halogenated derivatives are inactive (Nos. 42, 45), the ethyl ether is quite active (No. 43). This is interesting in view of the controversy about the necessity of having the hydroxyl group in position 8 free to get proper activity (13–18). 8-Acetoxyquinoline (No. 40) also shows very high activity.

While introduction of one nitro group in position 5 of 8-hydroxyquinoline (No. 59) imparts outstanding act vity against bacteria, two nitro groups (No. 60) decrease the activity. Halogenated mono-nitro hydroxyquinolines (Nos. 61, 62), on the other hand, are very promising. Introduction of an aldehydo or a keto group in position 5 greatly enhances the

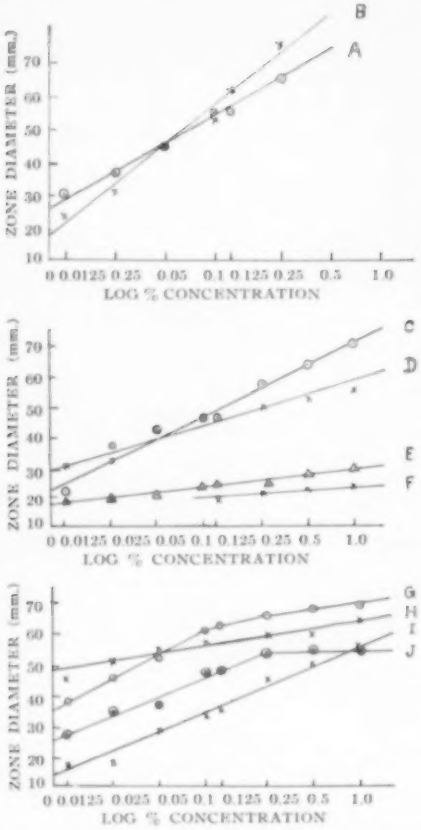


Fig. 1.—Dosage and fungistatic response curves of *A. niger* with increasing concentrations of 8-hydroxyquinoline derivatives. *A*, 8-hydroxyquinoline; *B*, 8-acetoxyquinoline; *C*, 5-acetyl-8-hydroxyquinoline; *D*, 5-ethyl-8-hydroxyquinoline; *E*, 5,7 dichloro-2-methyl-8-hydroxyquinoline; *F*, 5-chloro-7-iodo-8-hydroxyquinoline; *G*, 5-formyl-8-hydroxyquinoline, *H*, 5-nitro-8-hydroxyquinoline; *I*, 7-bromo-5-nitro-8-hydroxyquinoline; *J*, 7-bromo-5-formyl-8-hydroxyquinoline.

bacteriostatic activity, these compounds being very active against *A. niger* also (Nos. 46, 65, 66). With regard to further substitution in 5-formyl-8-hydroxyquinoline, a halogen group does not affect the activity while the introduction of a nitro group curtails the activity.

In the study of antibacterial spectra (Table II), 8-hydroxyquinoline itself shows wide activity but introduction of a nitro, aldehydo, or a keto group in position 5 gives compounds having still higher bacteriostatic as well as good fungistatic activity. Bromo-nitro- and bromo-formyl-hydroxyquinolines also exhibit high activity. As already stated, it is interesting that the compounds with an ethoxy or an acetoxy group in position 8 also show high bacteriostatic activity, particularly against the Gram-positive bacteria. It should be noted that all the compounds included in Table II also show good activity against *A. niger* by both the agar-streak and the agar cup-plate method.

The graphs giving the dosage-response curves of the most active compounds (Fig. 1) show that in the concentration range tested, most of them continue to exhibit a straight line response. However, 5-formyl-8-hydroxyquinoline and 7-bromo-5-formyl-8-hydroxyquinoline seem to have reached the maximum activity at 0.1 and 0.25%, respectively, since at concentrations higher than these, the graphs became almost flat. It is noteworthy that though for all the rest of the compounds in this series the zone diameter continues to increase with concentration even up to 1%, the gradient of slopes obtained for 5-chloro-7-iodo-8-hydroxyquinoline and 5,7-dichloro-2-methyl-8-hydroxyquinoline is low from the beginning.

Exceptionally high antiseptic activity of 8-acetoxyquinoline cannot be explained on the basis of chelation since the compound does not have a free hydroxyl group and is, therefore, nonchelating. Similarly, 8-ethoxyquinoline is quite active though nonchelating. The mode of action of these compounds may, therefore, be different from that of other 8-hydroxyquinoline derivatives. However, the stability of 8-acetoxyquinoline will have to be critically investigated.

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Studies on Quinoline Derivatives as Anti-infective Agents II

By S. M. BAHAL and M. L. KHORANA

Some quinoline derivatives, selected on the basis of previously reported screening, have been studied for their activity against *Epidermophyton floccosum*, *Trichophyton rubrum*, and *Trichophyton gypseum* by the agar cup-plate as well as by the agar-streak methods. Other tests conducted included the effect of organic matter on their activity and the *in vitro* activity when the compounds are incorporated in an ointment base. The compounds were finally tested *in vivo* for their interference in the normal rate of healing of wounds. It appears that 8-acetoxyquinoline, 5-formyl-8-hydroxyquinoline, and 5-acetyl-8-hydroxyquinoline might prove to be useful as antifungal agents.

RESULTS of preliminary screening of a number of quinoline derivatives for their antibacterial as well as antifungal activities have been communicated earlier (1). A more detailed study of the compounds which were found promising is recorded now.

With regard to antifungal spectra, a review of literature revealed that fungus species most prevalent in skin infections in different regions of the world differed considerably (2). Caro (3) had reported *Trichophyton gypseum* and *Trichophyton rubrum* to be the most common organisms. In India too, Srivastava and Gupta (4) showed the same two fungi to be the most prevalent cause of skin infections in the Lucknow area but in Bengal, Dutta, *et al.* (5), found *Epidermophyton floccosum* to be responsible for about 60 per cent of the infections. These three species *viz.*, *T. rubrum*, *T. gypseum*, and *E. floccosum* were, therefore, used for a study of antifungal spectra.

The compounds that showed satisfactory activity against the human pathogenic fungi were subjected to further tests under conditions simulating those in actual infections. These tests included (*a*) the effect of organic matter on the activity of the compounds, (*b*) *in vitro* activity when incorporated in an ointment base, and (*c*) *in vivo* interference in the normal process of epithelization of wounds.

EXPERIMENTAL

Antifungal Activity Against Dermatophytes.—*Agar Cup-Plate Method.*—The procedure adopted was the same as that used earlier for *Aspergillus niger* (1). The dermatophytes used were *T. rubrum*, *T. gypseum*, and *E. floccosum*. The concentrations of the test substances used were 0.5, 0.25, and 0.1% w/v. Average zone diameters of complete inhibition of growth at 0.25% concentration after incubation at 25–27° for seventy-two hours are given in Table I; other readings have been omitted for the sake of brevity.

Agar Streak Method.—Here too, following the procedure reported earlier (1), the concentrations used were 200, 100, 50, 20, 10, 5, and 1 mcg./cc. The minimum inhibition concentrations in mcg./cc. after incubation at 25–27° for five days are recorded in Table I.

Effect of Organic Matter.—The promising antifungal compounds selected from the above tests were studied by the agar-streak method using Sabouraud's agar medium containing 10% horse serum as the source of organic matter. The rest of the procedure was followed as described under "Agar Streak Method."

Activity in the Form of an Ointment.—Hydrophilic base recommended for sterilized cream of penicillin, "British Pharmacopoeia 1948," was used for preparing the ointments. The activity of different ointments containing the test substance in concentrations of 5, 2, 1, 0.5, 0.2, and 0.1% w/w, was tested by the agar cup-plate method using *T. gypseum* as the test organism. The average zone diameters of complete inhibition of growth after incubation at 25–27° for ninety-six hours are recorded in Table II.

Wound Healing Tests.—For studying the extent of interference in the normal process of epithelization of wounds, the procedure described by Dodd, *et al.* (6), was followed except that healthy adult male rats with an average body weight of 150 Gm. were used instead of rabbits. For each concentration of a compound, the backs of two to three rats were shaved and, on the next day, two excisions at the same level but on opposite sides of the dorsal midline were made with sterile scissors. The areas of the wounds were determined by tracing on cellophane paper. One of the wounds was treated with plain base, serving as a control, and the other with the test ointment. The wounds were covered with sterile lint and bandaged. On alternate days sizes of the wounds were recorded after removing any encrustations formed, and the animals were rebandaged after application of the fresh base or ointment. Healing was considered complete when epithelium first became continuous. The data are reported in Table III.

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DISCUSSION

The results of testing against pathogenic fungi by agar cup-plate method (Table I) showed that 8-acetoxyquinoline, 5-formyl-8-hydroxyquinoline, 5-acetyl-8-hydroxyquinoline, and 5-nitro-8-hydroxyquinoline had activity quite comparable to that of 8-hydroxyquinoline; they gave inhibition zones bigger than those of the parent compound in some cases and comparable in others. It is interesting to note that the two commonly used anti-infective agents 5-chloro-7-iodo-8-hydroxyquinoline and 5,7-dichloro-2-methyl-8-hydroxyquinoline failed to give good zones, indicating that they had poor penetration power.

Agar streak method in the absence of serum showed that most of the compounds were fungistatic for all three dermatophytes at 50 mcg./cc. 8-Acetoxyquinoline was slightly more active than 8-hydroxyquinoline and remained so even in the presence of serum. By this method 5,7-dichloro-2-methyl-8-hydroxyquinoline had activity comparable to that of 8-acetoxyquinoline in the absence of serum; in the presence of serum, the activity was affected adversely. The fungistatic activity of the other compounds tested was also affected by the presence of serum, though the effect could not be considered very damaging; most of the compounds were still fungistatic at 50 mcg./cc. against all three fungi.

Zones obtained with ointments (Table II) showed 8-acetoxyquinoline to be an effective fungistatic agent which possessed an outstanding power of penetration. It gave clear plates even at a concentration of 0.2%. The other promising compounds were 8-hydroxyquinoline, 5-acetyl-8-hydroxyquinoline, 5-formyl-8-hydroxyquinoline, 5-ethyl-8-hydroxyquinoline, and 5-nitro-8-hydroxyquinoline. The results also indicated that up to 0.5% of the compound in the ointment, the zone diameter increased with the concentration while above that, the increase in the concentration, 1 to 5%, did not produce corresponding increase in the zone diameter. It is noteworthy that 5,7-dichloro-2-methyl-8-hydroxyquinoline failed to come up to any of the other compounds reported.

Data on wound healing tests (Table III) showed that at 0.5% concentration, the two nitro derivatives, *viz.*, 5-nitro-8-hydroxyquinoline and 7-bromo-5-nitro-8-hydroxyquinoline and 7-bromo-5-formyl-8-hydroxyquinoline, slightly interfered with the normal rate of healing, delaying the process of epithelization by two days. The other compounds tested permitted normal healing. It was clear that the variation in the size of the wounds, within certain limits, did not affect the healing time, which was around thirteen days.

TABLE I.—FUNGISTATIC ACTIVITY OF SELECTED COMPOUNDS[a] AGAINST DERMATOPHYTES[b]

No.[c]	Compound	Agar Cup-Plate Method (Zone Diameter with 0.25% Solution, mm.)			Agar-Streak Method (Minimum Inhibition Concentration, mcg./cc.)					
					Without Serum			With 10% Serum		
		E. flocosum	*T. rubrum*	*T. gypseum*	*E. flocosum*	*T. rubrum*	*T. gypseum*	*E. flocosum*	*T. rubrum*	*T. gypseum*
37	8-Hydroxyquinoline	50	66	70	10	20	10	10	20	10
38	2-Methyl-8-hydroxyquinoline	15	26	27	20	50	50	100	100	100
39	5-Ethyl-8-hydroxyquinoline	24	41	40	20	20	20	50	20	20
40	8-Acetoxyquinoline	69	68	61	10	5	5	10	5	5
46	5-Acetyl-8-hydroxyquinoline	57	60	57	50	20	20	50	50	50
47	5-Acetyl-8-benzoyloxyquinoline	39	22	21	20	20	20	50	50	50
54	5-Chloro-7-iodo-8-hydroxyquinoline	19	19	17	100	100	50	100	100	100
55	5,7-Dichloro-2-methyl-8-hydroxyqinoline	29	29	25	10	10	10	50	50	50
59	5-Nitro-8-hydroxyquinoline	44	35	41	20	20	10	50	20	20
61	7-Bromo-5-nitro-8-hydroxyquinoline	46	31	32	50	50	50	100	100	100
65	5-Formyl-8-hydroxyquinoline	55	50	52	50	20	20	50	50	50
66	7-Bromo-5-formyl-8-hydroxyquinoline	50	27	26	50	20	50	50	50	50
69	5-Benzoyl-8-hydroxyquinoline	24	20	19	50	50	50	100	100	100
70	5-Benzyl-8-hydroxyquinoline	17	18	17	20	20	20	50	50	50

[a] Having minimum inhibition of less than 50 mcg./cc. against all the dermatophytes.
[b] Cultures of the test organisms were obtained from the Armed Forces Medical College, Poona, India.
[c] Numbers refer to compounds listed in the previous communication (1).

TABLE II.—FUNGISTATIC ACTIVITY OF OINTMENTS CONTAINING SELECTED 8-HYDROXYQUINOLINE DERIVATIVES AGAINST *Trichophyton gypseum*

Compounds[a]	Zone Diameters of ointments, mm. (Concentrations of test substances, % w/w)					
	5	2	1	0.5	0.2	0.1
37	[b]	[b]	[b]	[b]	80	60
39	54	51	48	32	30	22
40	[b]	[b]	[b]	[b]	[b]	53
46	61	59	56	51	47	42
55	30	27	27	25	None	None
59	38	36	35	33	27	24
61	31	29	29	24	23	21
65	45	43	40	39	30	27
66	28	26	26	25	20	18

[a] Numbers refer to compounds listed in Table I.
[b] Indicates no growth in 90-mm. Petri dishes.

TABLE III.—WOUND HEALING TESTS WITH SELECTED 8-HYDROXYQUINOLINE DERIVATIVES

Compound[a]	Test Substance in Ointment, %	Area of Unhealed Wounds, sq. mm., after 0 days	2 days	4 days	6 days	8 days	10 days	12 days	14 days	16 days
37	1	71	32	26	19	13	3	..	..	..
	c[b]	52	26	19	13	11	2	..	..	..
39	1	66	54	35	28	13	6	..	..	..
	c	51	42	32	23	7	2	..	..	..
40	1	58	50	40	26	13	3	..	..	..
	c	53	48	39	24	15	3	..	..	..
46	1	68	60	58	39	29	20	5	..	..
	c	89	61	42	26	16	11	6	..	..
	0.5	61	55	51	42	29	19	13	..	..
	c	77	52	32	29	19	16	10	..	..
55	1	65	61	58	52	29	13	5	..	..
	c	58	39	29	19	10	8	5	..	..
59	1	69	58	45	33	32	27	16	6	3
	c	60	48	35	21	15	13	6	..	..
	0.5	80	77	65	61	45	29	13	3	..
	c	48	35	23	19	15	10	3	..	..
61	1	61	58	55	42	26	13	10	6	..
	c	65	45	32	29	19	16	5	..	..
	0.5	68	55	48	35	23	10	6	..	..
	c	52	32	23	19	16	10	..	..	..
65	1	31	26	19	19	15	5	..	..	..
	c	37	31	21	18	11	6	..	..	..
66	0.5	68	52	35	23	16	11	5	..	..
	c	48	32	23	18	11	3	..	..	..

[a] Numbers refer to compounds listed in Table I. [b] c Indicates control.

The high activity shown by 8-acetoxyquinoline, where the hydroxyl group is blocked, is unexpected. However, it has not been definitely established that the activity is not due to the products of hydrolysis. The stability of the compound in pharmaceutical preparations would need further investigation. It may also be noted that even if the compound is hydrolyzed, none of the products of hydrolysis interfere with the normal rate of epithelization.

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Absorption of Tritiated *d*-Desoxyephedrine in Sustained-Release Dosage Forms

By J. F. NASH and R. E. CRABTREE

Randomly labeled H^3-*d*-desoxyephedrine was rigorously purified, and the stability of the label was established both *in vitro* and *in vivo*. This tracer compound was then combined with two types of cation exchange resins and administered in capsules to dogs. Both forms of drug resinates provided sustained release, but the rate of release for the carboxylic acid type was greatly influenced by the acidity of the medium. The use of an enteric coating (cellulose acetate phthalate, CAP) on the capsules delayed the start of absorption for three to four hours.

No TRULY satisfactory method is known for measuring anorectic activity of drugs in prolonged dosage forms. Human studies are usually of two sorts; disintegration and solubilization of the product in the gastrointestinal tract are observed with X-rays, or patients exhibit subjective responses, i. e., appetite suppression, central nervous system stimulation, etc., which are related to onset and duration of drug effect. The first method measures no drug level or response, and the second is dependent on the inherent error of subjective data.

Excellent reviews have appeared recently on the subject of extended action dosage forms (1, 2) and a number of methods of measurement are cited. The best one would be dynamic measurement of the drug concentration at the site of action. Since this is impractical in most cases, blood levels are used as a substitute.

In the experiments to be described, tritium-labeled *d*-desoxyephedrine in combination with resins was administered to dogs and the prolongation effects were observed. The experiments were designed to simulate conditions in patients who would take the drugs for weight control. Low doses (4 mg./dog) were used; the dose was administered after an overnight fasting period, and after a "light breakfast." Blood levels of the label were studied for twelve hours thereafter.

MATERIALS AND METHODS

Preparation and Purification of Tritiated *d*-Desoxyephedrine Hydrochloride.—Tritiation of *d*-desoxyephedrine hydrochloride was carried out by New England Nuclear Corp. following the Wilzbach (3) procedure. Labile tritium was removed by dissolution of the product twice in isopropanol which was removed *in vacuo*. The product was then stated to have 11 mc. of tritium in 0.913 Gm.

Purification was achieved by several crystallizations from hot isopropanol, transformation of the hydrochloride to the free base with sodium hydroxide solution, followed by reprecipitation of the hydrochloride salt from Skellysolve B solution with dry gaseous hydrochloric acid, and finally, by fractionation of the hydrochloride through use of partition chromatography. The column contained water as the stationary phase held on Whatman No. 1 cellulose powder; the mobile phase was chloroform.

Changes in specific activity were not used as the primary guide to purity since paper chromatography was found to give a much more accurate picture. The various fractions were spotted on Whatman No. 1 paper. Butanol saturated with dilute citric acid solution was used as the developer. When dry, the paper was sectioned into 1 cm. × 2.5 cm. segments which were bathed overnight in 1 ml. of methanol contained in 20-ml. counting vials. Liquid scintillation counting solution[1] was then added and the samples were counted in the Packard Tri-Carb (model 314X) liquid scintillation spectrometer. Figure 1 shows the initial chromatogram obtained with the crude tritiated material, and Fig. 2 shows the chromatogram of the pure product. The R_f value of 0.67 corresponded exactly to that of authentic *d*-desoxyephedrine. The leading component, R_f 0.90, moved at the same rate as the saturated analog, 1-cyclohexyl-2-monomethyl-aminopropane.

The specific activity of the final product was 3.4 μc./mg. The chemical yield was 35.4% (0.323 Gm.) and radioactivity yield 9.9%. In order to check the stability of this label, a portion of it was dissolved in water and stored for six months at room temperature. The radiochromatogram of the sample after six months appeared to be identical to the one shown in Fig. 2 with no trace of decomposition or tritium loss.

Other checks used to identify the product as *d*-desoxyephedrine hydrochloride were: X-ray diffraction, mixed m. p. with known drug, and Craig countercurrent distribution. In each method the product appeared to be pure H^3-*d*-desoxyephedrine hydrochloride.

Preparation of *d*-Desoxyephedrine Resinates.—The resins used were Amberlite IRC-50 and Amberlite IR-120,[2] both refined by cycling into the sodium phase and back to the hydrogen phase followed by thorough washing with distilled water.

H^3-*d*-Desoxyephedrine was prepared as the free base by making an aqueous solution of the hydrochloride (0.220 Gm. of 0.34 μc./mg. activity)

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[1] Diphenyloxazole (PPO) 0.5%, and 0.01% 2,2′-*p*-phenylenebis-(5-phenyloxazole) (POPOP) toluene solution.

[2] Rohm and Haas Co.

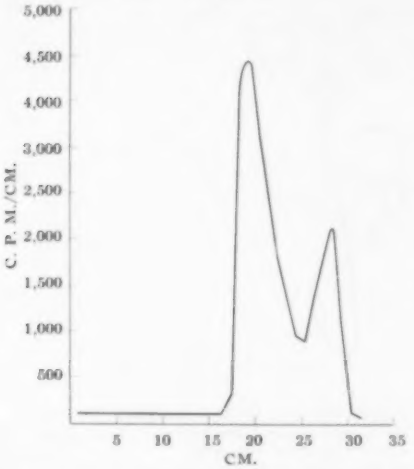

Fig. 1.—Paper chromatogram of H^3-*d*-desoxyephedrine hydrochloride before purification.

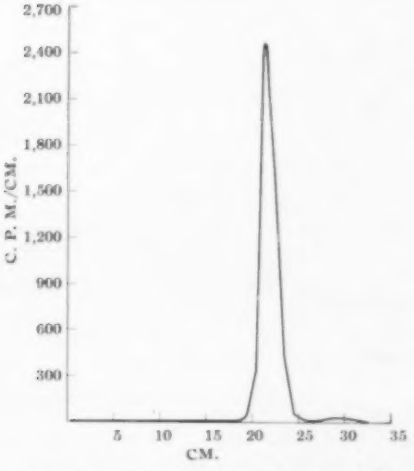

Fig. 2.—Paper chromatogram of purified H^3-*d*-desoxyephedrine hydrochloride.

strongly basic with sodium hydroxide and extracting the oil with Skellysolve B. The Skellysolve B was then removed *in vacuo*. The oil was finally dissolved in 50% ethanol-water (5 ml.).

Approximately one-half of this radioactive solution was then added to 400 mg. of each of the resins and shaken for twenty-four hours. The amine resinates were collected on Whatman No. 1 paper and washed continuously with 50% ethanol-water until the loosely bound drug had been removed. A constant low level of radioactivity in the filtrate was reached after 25–30 ml. wash. The products were dried forty-eight hours *in vacuo* over anhydrous calcium chloride.

For the determination of the amount of drug combined with resin, an aliquot (~2 mg.) was covered with 1 ml. of 10% hydrochloric acid and set aside overnight. To the resin mixture was added 0.5 ml. of 40% sodium hydroxide and 10 ml. of toluene scintillator solution. The mixture was thoroughly shaken and 2 ml. of the organic phase was counted in 10 ml. of additional counting solution. The count rate of the IRC-50 resinate indicated that 0.264 mg. of H^3-*d*-desoxyephedrine had combined with each mg. of the resin; for the IR-120 resinate 0.240 mg. combined per mg. of resin.

Capsules of the drug resinates and *d*-desoxyephedrine hydrochloride were prepared so that each capsule would contain the equivalent of 4 mg. of H^3-*d*-desoxyephedrine. A portion of these capsules were enteric coated with cellulose acetate phthalate (CAP).

Animal Experiments.—A Latin square experimental design was followed. Six healthy, female, mongrel dogs, each weighing approximately 10 Kg. were fasted overnight. Ten to twenty minutes before dosing, each dog was fed 100 Gm. of Pard dog food to insure an acid stomach. One capsule containing 4 mg. of H^3-*d*-desoxyephedrine was then given orally, followed by 100–200 ml. of water via a stomach tube. Blood samples of 1 ml. were then withdrawn from the cephalic or saphenous veins at one, two, three, four, six, eight, and twelve hours.

Two dogs per day were used, with a rest period of at least six days between series to allow for complete elimination of tritium.

Radioactivity Assays.—Blood samples were counted in a liquid scintillation counting solution adapted from one devised by Kinard (4). This solution, hereafter called "diotol," consists of dioxane, 500 ml.; toluene, 500 ml.; methanol, 300 ml.; naphthalene, 106 Gm.; PPO, 6.5 Gm., and POPOP, 130 mg. Shortly after withdrawal of the blood into a heparinized syringe, the large gauge needle was replaced with a 26-gauge one, and the blood was rapidly sprayed into 14 ml. of diotol, stirred well, and centrifuged. The clear supernatant was decanted and fresh diotol added to the dregs for a second extraction. Both samples were then counted for thirty minutes. Background samples were prepared from blood of the same dog taken before dosage. Internal standard was added to each sample to correct for quenching. Samples so prepared counted with about one-fourth the efficiency of toluene standards, i. e., approximately 6% absolute efficiency. To check the efficiency of extraction of H^3-*d*-desoxyephedrine from blood samples, a known amount of the labeled drug (1.03×10^5 c. p. m.) was added to heparinized dog blood and mixed thoroughly. Nine 1-ml. samples were sprayed into diotol as described above. The samples had a mean count of 9,980 c. p. m./ml. which represents 96.8% recovery of the tritium added. The range was 95.1 to 98.7% recovery.

Stability of the Label in Vivo.—In order to establish the validity of using the tritium-labeled material as a tracer compound for *d*-desoxyephedrine, it was necessary to prove that the radioactivity in the urine of the animals was attached only to the expected metabolities of *d*-desoxyephedrine, and had not exchanged with other normal constituents of the body.

Sodium hydroxide (10 Gm.) was added to 500 ml. of urine collected from the dogs during twenty-four-hour periods. This solution was reduced in volume

by distillation *in vacuo* over a steam bath. A count of the distillate revealed that an insignificant amount of H^3 was carried over. This proved that there was little or no exchange with body water.

This alkaline urine concentrate was extracted with an equal volume of benzene which was then evaporated at room temperature under a gentle stream of air. Countercurrent distribution was run on the dry residue with benzene-1 *M* phosphate buffer, pH 8.0. This was the system used by Axelrod (5) to study the metabolites of amphetamine and desoxyephedrine. The radioactivity pattern can be seen in Fig. 3. The benzene layers of the two main peaks were combined and evaporated, pure H^3 *d*-desoxyephedrine was added, and this mixture was again submitted to countercurrent distribution. The results are shown in Fig. 3. It was evident that much of the H^3 in the urine was in the form of a compound quite different in distribution from *d*-desoxyephedrine.

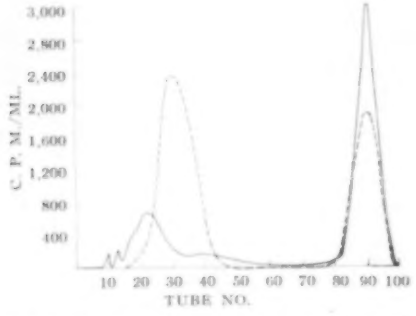

Fig. 3.—Countercurrent distribution of dog urine extract. ———, Initial results; - - - - - - results after addition of known H^3-*d*-desoxyephedrine.

In order to check for phenylacetone as an oxidative metabolite (5) the leading distribution peak was removed and evaporated; the residue was allowed to react with 2,4-dinitrophenylhydrazine. The product was analyzed by passage through a bentonite-silica gel column in chloroform, and then paper chromatography of the radioactive fraction was run in butanol-water. The R_f value corresponded exactly to that of the authentic hydrazone of phenylacetone. Thus, the H^3-compound in the urine was identified as a known metabolite of *d*-desoxyephedrine.

RESULTS

In Figs. 4, 5, and 6 the mean levels of tritium in the blood from the various dosage forms are shown. The standard deviations of the means for the six dogs are also shown. When H^3-*d*-desoxyephedrine hydrochloride was given, the tritium in the blood reached a peak within two hours. The CAP-coated capsules of the free hydrochloride gave negligible levels of H^3 until three to four hours after dosage; then an abrupt rise occurred, reaching a peak at six hours. Apparently, this period of time was required for the capsule to be passed through the pyloric sphincter of the dog and to be dissolved in the higher pH of the intestinal juice.

The uncoated H^3-*d*-desoxyephedrine Amberlite

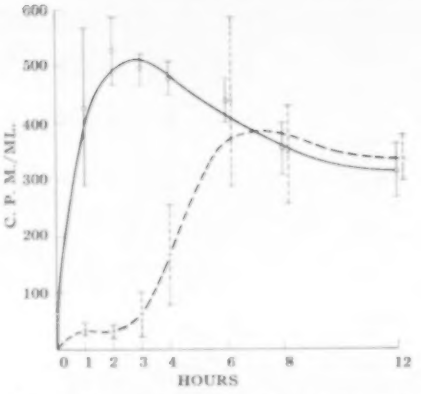

Fig. 4.—Tritium blood levels after administration of H^3-*d*-desoxyephedrine hydrochloride to six dogs. ———, Uncoated capsules; - - - - - - CAP-coated capsules. Bars show the standard deviations of the means.

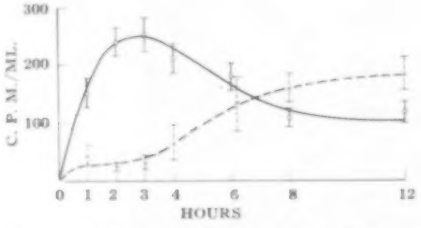

Fig. 5.—Tritium blood levels after administration of H^3-*d*-desoxyephedrine-IRC-50 resinate to dogs. ———, Uncoated capsules; - - - - - -, CAP-coated capsules. Bars show the standard deviations of the means.

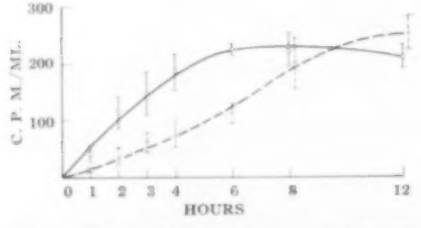

Fig. 6.—Tritium blood levels after administration of H^3-*d*-desoxyephedrine-IR-120 to dogs. ———, Uncoated capsules; - - - - -, CAP-coated capsules. Bars show the standard deviations of the means.

IRC-50 gave results similar to the uncoated hydrochloride salt except that the levels were approximately one-half those obtained with the hydrochloride. The CAP-coated IRC-50 resinate gave insignificant H^3 levels until four hours after dosage. Then a gradual increase occurred through at least twelve hours.

The uncoated capsules of H^3-*d*-desoxyephedrine Amberlite IR-120 behaved differently. In the case

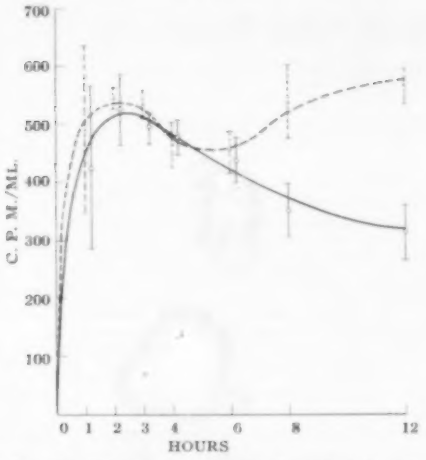

Fig. 7.—Tritium blood levels after administration of H^3-*d*-desoxyephedrine to dogs. ———, Hydrochloride salt only; – – –, hydrochloride salt plus IRC-50 resinate (CAP-coated). Bars show the standard deviations of the means.

of the IR-120 resinate a gradual rise in drug blood level occurred in almost a straight-line relationship with time, reaching a plateau after six hours. Coating the IR-120 resinate with CAP delayed this rise for approximately three to four hours, after which the same gradual rise in blood levels occurred.

Five of the dogs were given a further treatment to establish the effect of a combined dosage of the *d*-desoxyephedrine hydrochloride and the *d*-desoxyephedrine Amberlite IRC-50 as follows: capsules of the uncoated H^3-*d*-desoxyephedrine hydrochloride and the CAP-coated H^3-*d*-desoxyephedrine Amberlite IRC-50 were administered simultaneously. The procedure followed was the same as that previously described. The dogs were used in a different order from that previously used in order to provide some randomness in the order of treatment.

The H^3 blood levels obtained with this combined dosage are shown in Fig. 7. The experimental levels during the first hours were almost identical to the levels obtained when the hydrochloride salt was administered alone. No statistically significant difference between the blood levels for the combined dosage and the hydrochloride salt was found for the first six hours. However, the combined dosage levels at eight and twelve hours were considerably higher than, and significantly different from, the levels obtained with the hydrochloride administered alone. At the eighth hour, $p < 0.05$, at the twelfth hour, $p < 0.01$.

DISCUSSION

This study was undertaken in order to determine the rate of drug release *in vivo* from two different ion exchange resins; Amberlite IRC-50, a carboxylic acid resin, represented the weak acid type and Amberlite IR-120, a sulfonic acid resin, was a strong acid type. In addition, the effect of an enteric coating, CAP, on the rate of drug release was examined. The blood levels of tritium were assumed to indicate the drug release and absorption.

As was expected, in the stomach the drug was rapidly removed from the carboxylic acid resin. This is because the equilibrium of a weak acid at the pH of the stomach favors the unionized form. On the other hand, in the stomach the drug was removed more slowly from the sulfonic acid resin because its equilibrium favors the ionized form at all biological pH values. In the case of the sulfonic acid resin, drug removal depends primarily on the ionic strength and electrostatic charge of the cations competing with the drug for anionic sites.

The enteric coating on the capsules delayed the start of drug removal until the capsule reached the duodenum or upper intestinal tract. CAP dissolves only at the pH higher than 5. At the normal pH of the intestine both resins behave as ion exchange media, the equilibria favoring the ionized state. The rate of drug removal, therefore, depends on ionic strength. The cations of the intestinal contents compete with the drug for anionic sites on the resin. This reaction is relatively slow and therefore results in a sustained release of drug.

In these experiments significant tritium levels were found in the blood twelve hours after dosage. Axelrod (5) measured the blood levels of amphetamine in dogs by a color reaction with methyl orange. He also found prolonged levels and estimated that the plasma levels decline at the rate of 8% per hour. There probably are tissue deposits of the drug from which it gradually diffuses back into the blood stream, prolonging the blood levels for many hours. In addition, Axelrod found that low dosages, such as the ones used in these experiments, are more slowly excreted than high doses.

The relative merits of the various dosage forms as appetite suppressants depend upon the type of effect desired. Should the drug be released slowly at first, reaching a peak at about the time of the next meal, or should it have a fast onset and remain at peak level the rest of the day? If the first type of effect is desired, then dosage of the IR-120 drug resinate (uncoated) would give the desired effect. If the second type is desired, then dosage of the free hydrochloride plus an inner core of either of the drug resinates coated with CAP would give the effect.

SUMMARY

1. Tritium-labeled *d*-desoxyephedrine was prepared and its suitability for an animal tracer experiment was established.

2. H^3-*d*-Desoxyephedrine combined with a sulfonic acid or carboxylic acid type of ion exchange resin was shown *in vivo* in dogs to provide sustained release.

3. Enteric coating (CAP) delayed the absorption for three to four hours.

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(4) Kinard, F. E., *Rev. Sci. Instr.*, **28**, 293(1957).
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Interaction of Sorbic Acid with Nonionic Macromolecules

By SEYMOUR M. BLAUG and SAYED S. AHSAN

Sorbic acid was shown to interact with various nonionic macromolecules. The presence of a nonpolar moiety in the nonionic contributed greatly to the degree of interaction. Thus, Tween 80 and Myrj 52 interacted with sorbic acid to a much greater degree than did polyethylene glycols 4000 and 6000 and Pluronic F-68. Tween 80 was shown to have an inhibitory effect on the preservative action of sorbic acid. However, in the concentrations normally used for preservation it was shown that there would be little or no interference with the preservative action of sorbic acid in the presence of Tween 80. In the presence of the polyethylene glycol or polyethylene polypropylene glycol-type of macromolecule there would be even more free sorbic acid to act as a preservative because of the lesser degree of interaction between sorbic acid and these macromolecules.

SORBIC ACID[1] has been used as a preservative for many years. Chemically it can be described as 2,4-hexadienoic acid (CH_3—CH=CH—CH=CH—COOH). It is a white crystalline material that melts at 134.5°. It is slightly soluble in water (0.18% w/w at 25°). Although the chemical has its own characteristic odor, it does not usually impart this to the product in which it is incorporated (1). The physical properties of sorbic acid and its activity against various microorganisms have been reported by deNavarre (2).

Some of the early work using sorbic acid in pharmaceutical preparations was conducted by Cosgrove, *et al.* (3). Their experiments were designed to investigate the fungistatic activity of sorbic acid, since it was this property that Melnick, Luckmann, and Gooding (4) reported. The results of the pharmaceutical study showed that sorbic acid could be used to better advantage than benzoic acid as a fungistatic agent in certain pharmaceutical preparations.

The preservation of pharmaceutical formulations containing nonionic macromolecules has been studied by deNavarre (5, 6). He reported that sorbic acid was ineffective as a preservative in the presence of Tweens and other nonionic macromolecules. Barr and Tice (7) reported that sorbic acid, in a concentration of 0.10–0.20 per cent w/v, was effective as a preservative for solutions containing nonionic surfactants of the sorbitan partial fatty acid and polyoxyethylene ester types.

The discrepancy in the conclusions reached by deNavarre and Barr and Tice may be due to the effect of the medium itself on the growth of the microorganism. deNavarre conducted his experiments using Jaag medium, which is an enriched synthetic medium. Barr and Tice used an aqueous system to determine the preservative activity of sorbic acid against several microorganisms. The degree to which sorbic acid interacts with nonionic macromolecules may be altered by the presence of electrolytes and organic molecules in the synthetic medium as well as by the difference in pH between an aqueous solution of sorbic acid and sorbic acid in a synthetic medium.

The effectiveness of sorbic acid as a mold inhibitor is attributed to the fact that as an unsaturated fatty acid it inhibits the dehydrogenase enzyme system present in molds (4). This dehydrogenase enzyme system is responsible for the dehydrogenation of fatty acids to unsaturated compounds, a reaction which is vital to the growth and survival of the mold. The loss of this enzymatic process disrupts the metabolic processes of the mold causing it to diminish in population.

In a study conducted by Charles and Carter (8) on the effect of sorbic acid and other preservatives on organism growth in typical nonionic emulsified commercial cosmetics it was shown that sorbic acid provided adequate protection against organism growth in preparations inoculated with various organisms. This lends support to the contention that the preservation of nonionic cosmetic emulsions depends upon more factors than the interaction between preservative and nonionic surfactant.

It was the purpose of this study to investigate the interaction of sorbic acid with various nonionic macromolecules in aqueous solutions. The effect of an enriched synthetic medium on the degree of interaction between sorbic acid and Tween 80[2] was also studied. The effect of Tween

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[1] Product of Union Carbide Chemicals Co., New York, N. Y.

[2] Polyoxyethylene sorbitan monooleate, Atlas Powder Co., Wilmington, Del.

80 on the preservative action of sorbic acid was investigated to see if any correlation exists between the degree of interaction and preservative concentration required to completely inhibit the growth of *Pseudomonas aeruginosa* and *Aspergillus niger*.

EXPERIMENTAL

Interaction of Sorbic Acid with Nonionic Macromolecules.—The procedure employed in this study was similar to the solubility method previously described by Higuchi and Lach (9). Complexing studies with the various nonionic macromolecules were conducted in 0.005 *N* sulfuric acid (to suppress any ionization of sorbic acid). If complexing takes place to an appreciable degree one observes either an increase or decrease in the solubility of the sorbic acid depending on the nature of the complex formed. The interaction of sorbic acid with Tween 80 was also studied in a synthetic culture medium in order to determine the influence of the synthetic medium on the binding of sorbic acid by Tween 80.

The interaction of sorbic acid with the various nonionic macromolecules in 0.005 *N* sulfuric acid and with Tween 80 in a synthetic culture medium is shown in Figs. 1 and 2. Data were plotted to show the ratio, *R*, of total sorbic acid in solution to the concentration of free sorbic acid as a function of the nonionic concentration. The relative complexing activity taking place in these systems can be compared by comparing the per cent increase in solubility of sorbic acid ($R \times 100$) at a definite concentration of complexing agent. The *R* value can also be used to approximate the total sorbic acid concentration which must be employed in a system containing a known concentration of nonionic agent because the *R* value multiplied by the required free sorbic acid concentration gives the total sorbic acid concentration which must be employed. For example, if 0.1% of sorbic acid is required in a formulation containing no Tween 80, then approximately three times that amount would be required in a formulation containing 5% Tween 80 in order to insure the presence of 0.1% unbound sorbic acid.

Since the concentration of the free sorbic acid remains constant, the stoichiometric ratio of the

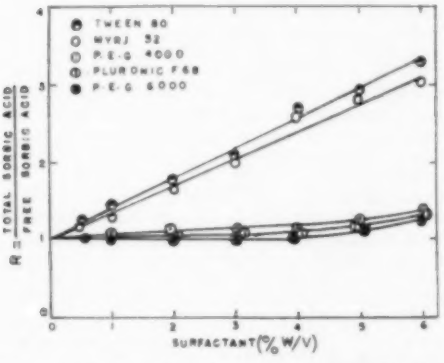

Fig. 1.—Binding of sorbic acid by several polyoxyethylene-type macromolecules in water at 30°.

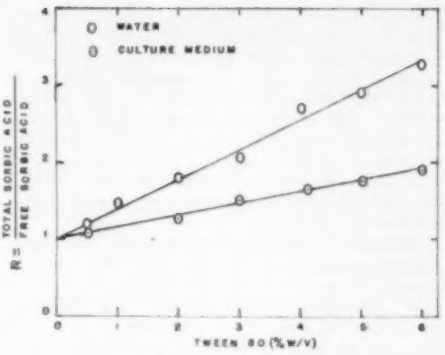

Fig. 2.—Binding of sorbic acid by Tween 80 in water and in the culture medium at 30°.

complexes formed cannot be determined from the phase diagrams, hence the true stability constants cannot be calculated. However, from the phase diagrams, it appears that the principal complex being formed is directly dependent on the nonionic concentration. The equilibrium between free and bound sorbic acid can be considered as follows:

$$\text{sorbic acid} + \text{nonionic} \rightleftharpoons \text{sorbic acid-nonionic}$$

Effect of Tween 80 on Inhibitory Concentration of Sorbic Acid.—The inhibitory concentration of sorbic acid for *Pseudomonas aeruginosa* and *Aspergillus niger* was determined in a synthetic medium and in the synthetic medium containing varying concentrations of Tween 80 using a procedure similar to the method previously described by Pisano and Kostenbauder (10).

PROCEDURE

Reagents.—Recrystallized sorbic acid, m. p. 132°, Tween 80, Myrj 52,[3] Pluronic F-68,[4] polyethylene glycols 4000 and 6000, 0.005 *N* sulfuric acid, 0.1 *N* alcoholic sodium hydroxide solution, bromothymol blue indicator, Sabouraud dextrose agar, trypticase soy agar, *Pseudomonas aeruginosa* ATCC No. 10145, and *Aspergillus niger* ATCC No. 6277. The following synthetic medium was used:

$NH_4H_2PO_4$	1 Gm.
NaCl	5 Gm.
$MgSO_4.7H_2O$	2 Gm.
$K_2HPO_4.3H_2O$	1 Gm.
Dextrose, anhydrous	5 Gm.
Water, to make	1,000 ml.

pH = 6.35

Solubility Method.—Excess quantities of sorbic acid were accurately weighed and placed in 125-ml. glass-stoppered bottles together with varying amounts of the nonionic macromolecules. Sufficient 0.005 *N* sulfuric acid solution was added to each bottle to bring the volume to 50 ml. Blanks were prepared containing varying concentrations of the nonionics but no sorbic acid. The bottles were

[3] Polyoxyethylene stearate, Atlas Powder Co., Wilmington, Del.
[4] Polyethylene polypropylene glycol, Wyandotte Chemicals Co., Wyandotte, Mich.

placed in a mechanical shaker in a constant temperature bath and equilibrated at 30 ±0.1° for twenty-four hours, after which time aliquot portions of the supernatant liquid were removed and analyzed for sorbic acid content by titration with alcoholic sodium hydroxide, using bromothymol blue indicator. A corresponding blank was titrated for each concentration of nonionic. The same procedure was used to determine the solubility of sorbic acid in the synthetic medium containing varying concentrations of Tween 80.

Preparation of Test Cultures.—The desired concentrations of Tween 80 and sorbic acid were dissolved in the synthetic medium with the aid of heat. Ten-milliliter portions were then transferred to 18 × 150 mm. Pyrex test tubes. The tubes were stoppered with nonabsorbent cotton plugs wrapped in gauze pads to prevent lint from falling into the medium. The plugs and upper portions of the tubes were wrapped with aluminum foil prior to sterilization. The following concentrations of sorbic acid were used: 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, and 0.15%. Each of these concentrations was run in the synthetic medium containing 0, 1.0, 3.0, and 5.0% Tween 80. The tubes were sterilized by autoclaving at 15 pounds pressure for fifteen minutes.

Pseudomonas aeruginosa was cultured on slants of trypticase soy agar for five days at 37.5°. A bacterial suspension was prepared by inoculating a test tube containing 25 ml. of the synthetic medium with two loopfuls of the culture. The culture-containing test tube was allowed to incubate for seventy-two hours at 37.5°. At the end of this period the previously prepared test tubes containing the sorbic acid and Tween 80 were inoculated with 0.1-ml. portions of the culture. Duplicate runs were made for each sorbic acid-Tween 80 concentration. Blank tubes containing sorbic acid but no Tween 80 and tubes containing Tween 80 but no sorbic acid were also inoculated. After inoculation, tubes were maintained at 37.5° for six weeks. Periodically, the tubes were examined visually to follow the growth pattern.

Aspergillus niger was cultured on slants of Sabouraud dextrose agar for two weeks at room temperature. A spore suspension was prepared by inoculating a test tube containing 25 ml. of the synthetic medium with two loopfuls of the fungus culture. The tube was incubated for five days at room temperature. The subsequent inoculation procedure was identical to the one outlined for *Pseudomonas aeruginosa*.

Table I.—Inhibitory Concentration of Sorbic Acid in the Presence of Tween 80[a]

Organism	Tween 80, %	Sorbic Acid, % — Predicted	Sorbic Acid, % — Experimental
Pseudomonas aeruginosa	0	.....	0.07–0.08
	1	0.0805–0.092	0.08–0.09
	3	0.105 –0.150	0.09–0.10
	5	0.120 –0.137	0.09–0.10
Aspergillus niger	0	.....	0.07–0.08
	1	0.0805–0.092	0.08–0.09
	3	0.105 –0.150	0.09–0.10
	5	0.120 –0.137	0.10–0.15

[a] Results obtained on duplicate runs after six weeks observation.

The results in Table I show the minimum inhibitory concentration of sorbic acid for *Pseudomonas aeruginosa* and *Aspergillus niger* as a function of Tween 80 concentration at the end of the six-week period. The predicted inhibitory concentrations were obtained by multiplying the inhibitory concentration of sorbic acid in the synthetic medium at 0% Tween 80 (0.07–0.08% sorbic acid) by the R value obtained from Fig. 2 at 1, 3, and 5% Tween 80 concentrations.

DISCUSSION

The binding tendency which sorbic acid exhibits for the nonionic macromolecules is attributable to the association of the acidic hydrogen in sorbic acid with electrophilic atoms like oxygen in the macromolecules. According to the data in Fig. 1, the presence of a nonpolar moiety in the nonionic (Tween 80 and Myrj 52) contributes greatly to the degree of interaction. Macromolecules such as polyethylene glycols 4000 and 6000 and Pluronic F-68 have electrophilic oxygen atoms to hydrogen bond with the acidic hydrogen in sorbic acid but contain no large hydrophobic groups. Therefore, they interact to a much lesser degree with sorbic acid than do Tween 80 and Myrj 52. In actual practice there should be little or no interference with the preservative action of sorbic acid in the presence of polyethylene glycols 4000 and 6000 and Pluronic F-68 especially since sorbic acid is usually used in concentrations in excess of the minimum required for complete inhibition.

From the results shown in Fig. 2 it can be seen that sorbic acid interacts with Tween 80 to a lesser degree in the synthetic medium than in distilled water. This is probably due to the difference in ionization of sorbic acid in the medium and in the 0.005 N sulfuric acid solution. The pKa of sorbic acid is 4.76 and in the synthetic medium containing varying concentrations of Tween 80 the pH of the sorbic acid solutions ranged between 4.98–5.70. At these pH's sorbic acid is more than 50% ionized whereas in the 0.005 N sulfuric acid solution it is undissociated.

The data in Table I indicate that Tween 80 does have an inhibitory effect on the preservative action of sorbic acid. However, since sorbic acid is usually used as a preservative in a concentration of 0.15 to 0.20% it can be seen that sufficient unbound sorbic acid would be present in systems containing 5% nonionic of the Tween type. In the presence of the polyethylene glycol or polyethylene polypropylene glycol type of macromolecule there would be far more free sorbic acid present because of the lesser degree of interaction between sorbic acid and these macromolecules. Where growth did occur (in tubes containing less than 0.07% sorbic acid) it was much heavier in the tubes containing Tween 80 than in those in which the Tween 80 was absent. The growth of *Pseudomonas aeruginosa* in the presence of Tween 80 and sorbic acid was studied at 37.5° and the interaction of sorbic acid with Tween 80 was studied at 30°. The incubation at 37.5° was necessary in order to obtain optimum growth of the microorganism.

Since the aqueous phase of a cosmetic or pharmaceutical emulsion seldom represents an enriched me-

dium, there should be little or no interference with the preservative action of sorbic acid (concentration of 0.15–0.20%) when used in commercial formulations containing emulsifiers of the Tween and Myrj types. The work of Charles and Carter tends to support this observation (8). However, cosmetic and pharmaceutical emulsions are complex systems and their preservation depends on many factors such as pH, electrolyte concentration, water-soluble organic material content, etc. All of these factors influence the degree of interaction between preservative and nonionic which may account for the difference in behavior of some preservatives in finished formulations than had been predicted from laboratory inhibition tests.

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Color Stability of Tablet Formulations III

Comparative Light Fastness of Several Water-Soluble Dyes and Their Corresponding Lakes

By L. LACHMAN, S. WEINSTEIN, C. J. SWARTZ, T. URBANYI, and J. COOPER

The relative light fastness of several certified water-soluble dyes and their corresponding lakes were investigated. The water-soluble dyes studied were FD&C Red No. 1, FD&C Blue No. 1, and FD&C Yellow No. 5. The lakes investigated consisted of water-soluble dyes precipitated on a suitable substrate and were D&C Red No. 1 lake, D&C Blue No. 1 lake, and D&C Yellow No. 5 lake. Tablets containing the above colorants were stored under normal and exaggerated illumination of similar spectral energy distribution. The apparent rates of fading of the several dyes were evaluated. The differences in light fastness between lakes and water-soluble dyes are discussed.

LAKES AS COLORANTS for pharmaceuticals have received considerable attention by many industrial laboratories. This intensified interest stems from recent FDA rulings (1) which certify lakes, prepared by extending the salts of water-soluble certified dyes on a substrate of alumina, as being suitable for food, drug, and cosmetic use. In addition to their newly approved status, lakes have been reported to have inherent pharmaceutical advantages. They have been considered to exhibit improved light stability, additional color versatility, and more color uniformity. Tucker, *et al.* (2), found that besides ease of color reproducibility and simplification of pan coating techniques, enhanced light stability resulted from the use of lakes as tablet colorants. Relative photosensitivity of several lakes used in pan coated tablets was determined after twenty-four-hour exposure to radiant energy from a G. E. type R. S. reflector sun lamp. In a previous article of this series (3) the photosensitivity of a number of commonly used certified water-soluble dyes was investigated following exposure to a newly established exaggerated lighting system. Because of the recent interest shown in certified lakes as tablet colorants, their inclusion in our studies involving the color stability of tablet formulations seemed appropriate.

A project was initiated to investigate the relative light fastness of several soluble dyes and their corresponding lakes. The fading at the surface of the tablets exposed to both normal and exaggerated illumination was measured by reflectance techniques and apparent fading rates for the dyes and lakes were compared

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EXPERIMENTAL

Materials Used.[1]—FD&C Red No. 1; D&C Red No. 1 lake containing 35 to 38% FD&C Red No. 1 precipitated on alumina hydrate; FD&C Blue No. 1; D&C Blue No. 1 lake containing 11–12% FD&C Blue No. 1 precipitated on alumina hydrate; FD&C Yellow No. 5; D&C Yellow No. 5 lake containing 16–18% FD&C Yellow No. 5 precipitated on alumina hydrate.

Equipment.—Light stability cabinets as described in a previous publication (4). Gossen Tri Lux foot candle light meter, model C. Stokes model F tablet press. Beckman spectrophotometer model DU with reflectance attachment, as described in a previous paper (5). Ball mill with porcelain balls. Precision rotary microtome.

Preparation of Tablets.—The concentration of dye in the lakes used for this study ranged from 11.5% for D&C Blue No. 1 to 36.5% for D&C Red No. 1. Preliminary tests indicated that 4% of lake was necessary to impart a color of adequate tinctorial power to the tablets so that an accurate study of the degree of fading could be made. To permit a valid comparative light stability study of tablets colored with water-soluble dyes and lakes of the same dye, the concentration of dye in the amount of lake used for the tablets was calculated, and the same quantity of dye was used for the tablets colored with the water-soluble dye. Representative formulas for the tablets colored with water-soluble dye and lake are as follows:

Lake	
Calcium sulfate, dihydrate	95%
Magnesium stearate U. S. P.	1%
Lake	4%

Dye	
Calcium sulfate, dihydrate	% = to bring up to 100% by wt.
Magnesium stearate U. S. P.	1%
Water-soluble dye	% = to the amt. of dye in 4% lake
Purified water	q. s.

Tablets colored with the lakes were prepared by intimately mixing the lake with the calcium sulfate dihydrate and magnesium stearate in a ball mill for one hour with one pound of porcelain balls. The mix was then slugged on a Stokes F tablet press. The slugs were then screened through a No. 16 screen and compressed into tablets on the same press using $^{16}/_{32}$-inch flat punches, upper bisected. The thickness of the tablets was kept at approximately 3.5 mm. with a weight of 860 mg. The moisture content of the granulation was below 0.5%.

For the tablets colored with the water-soluble dyes, the color was dissolved in the water which was subsequently used to granulate the calcium sulfate dihydrate. The dried granulation was screened, lubricated, and compressed into tablets as above. The moisture content of the dried granulation was maintained below 0.5%.

Storage of Samples in the Light Stability Cabinet.—Storage was similar to that described in a previous paper of this series (3). The light intensities falling on the tablets in the normal and exaggerated illumination cabinets were 45 and 550 foot candles, respectively. Tablet samples were withdrawn from the cabinets at designated time intervals for reflectance measurements.

Measurement of Tablet Reflectance.—The procedure and equipment employed to evaluate fading at the tablet surface were presented in previous publications (3, 5). Reflectance measurements were performed at 400 mμ for FD&C Yellow No. 5, at 410 mμ for D&C Yellow No. 5 lake, at 500 mμ for FD&C Red No. 1 and D&C Red No. 1 lake, at 640 mμ for FD&C Blue No. 1 and D&C Blue No. 1 lake.

[1] Dyes and lakes obtained through the courtesy of H. Kohnstamm and Co.

RESULTS AND DISCUSSION

The comparative light sensitivity of water-soluble dyes and their corresponding lakes when used as tablet colorants was investigated by exposure to normal and exaggerated illumination.

Figures 1 and 2 are representative plots showing a decrease in the spectral absorbance of the surface of the tablets colored with FD&C Blue No. 1 and D&C Blue No. 1 lake and FD&C Red No. 1 and D&C Red No. 1 lake. In each case a flattening of the absorption curve is noticed as fading progresses. In addition, the tablets colored with Blue lake and dye exhibit a significant hypsochromic shift of the absorption maximum. After forty-two days' storage, the tablets colored with FD&C Blue No. 1 and exposed to intensified illumination became practically colorless as indicated by the plateau effect beginning at 610 mμ of the absorption curve. The difference in absorbance along the plateau at forty-two and eighty-four days can be ascribed to the whitish-gray appearance of tablet surface as compared to the U. S. Bureau of Standards vitriolite plate. In contrast it is worthy to note that the tablets colored with D&C Blue No. 1 lake, although almost completely faded, retain the character of the lake's absorption curve after eighty-four days' exposure to exaggerated light intensity.

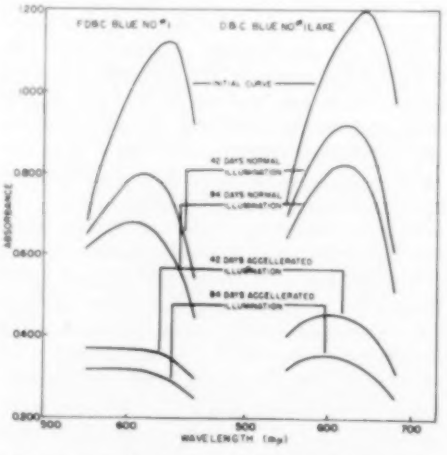

Fig. 1.—Plots of the visible absorption spectra of FD&C Blue No. 1 and D&C Blue No. 1 lake after different intervals of storage under normal and exaggerated illumination.

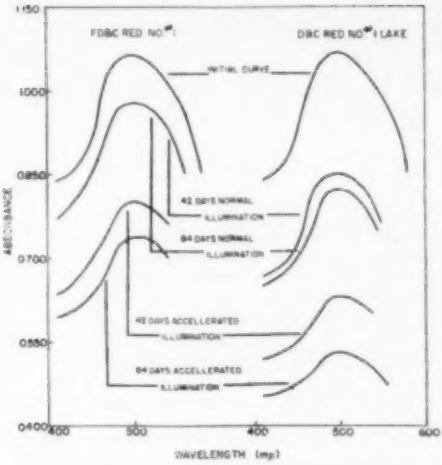

Fig. 2.—Plots of the visible absorption spectra of FD&C Red No. 1 and D&C Red No. 1 lake after different intervals of storage under normal and exaggerated illumination.

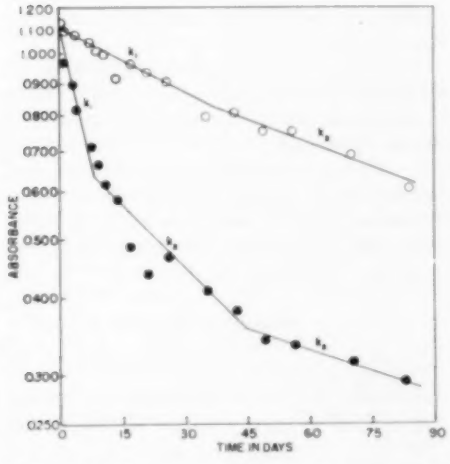

Fig. 3.—Influence of light intensity on the fading of the surface of tablets colored with FD&C Blue No. 1. ○, Ordinary light; ●, accelerated light.

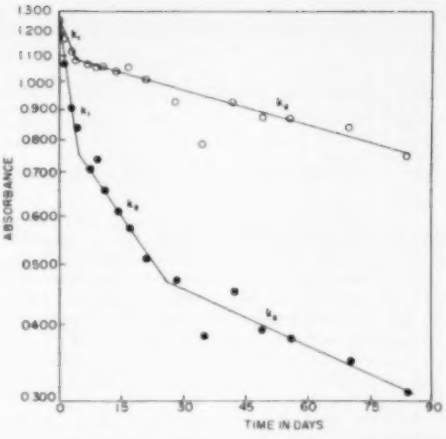

Fig. 4.—Influence of light intensity on the fading of the surface of tablets colored with D&C Blue No. 1 lake. ○, Ordinary light; ●, accelerated light.

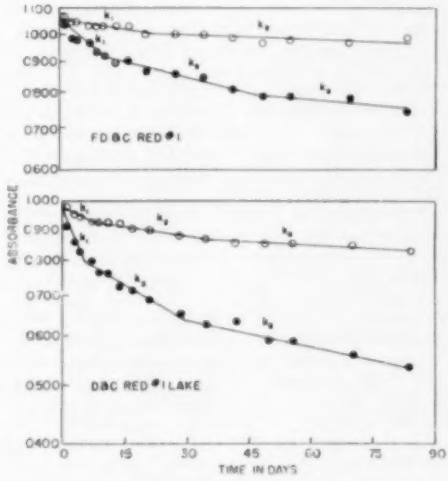

Fig. 5.—Influence of light intensity on the fading of the surface of tablets colored with FD&C Red No. 1 and D&C Red No. 1 lake. ○, Ordinary light; ●, accelerated light.

Plots showing the rates of fading for the tablets colored with the various dyes and lakes are presented in Figs. 3–5. The linearity of these plots indicates that the fading is proceeding according to an apparent first-order reaction. It is evident from these plots that the curves show more than one slope. In all cases, the initial rate of fading was greater than that obtained in subsequent sections of the fading curve. These data are similar to those found in our previous study (3) and to those found by Baxter, *et al.* (6), in their studies of fastness of dyes in textiles.

The changes in reaction rates that are observed as the duration of irradiation is increased do not necessarily indicate that the fading mechanism consists of a series of consecutive reactions of different rates. Instead they are most likely due to several contributing factors which have already been discussed in detail (3).

In Table I the rate constants for the fading of the several colored tablets are presented. Although the table contains the rate constants for the different slopes of each curve only the initial rate, k_1, will be used in subsequent discussions to represent thecolor stability at the tablet surface.

While it has been generally accepted that lakes

TABLE I.—RATE CONSTANTS FOR THE FADING OF COLOR IN COMPRESSED TABLETS IN DAYS^{-1}

Color	Ordinary Lighting			Accelerated Lighting		
	$K_1 \times 10^3$	$K_2 \times 10^3$	$K_3 \times 10^3$	$K_1 \times 10^3$	$K_2 \times 10^3$	$K_3 \times 10^3$
FD&C Red No. 1	1.75	0.51	...	12.8	3.99	1.53
D&C Red No. 1 lake	8.43	2.73	0.992	38.4	9.05	3.29
FD&C Blue No. 1	6.13	6.05	...	69.0	16.40	5.29
D&C Blue No. 1 lake	43.00	4.60	...	108.0	24.00	7.18
FD&C Yellow No. 5	0.27	...	...	6.1	1.35	...
D&C Yellow No. 5 lake	3.06	2.25	...	32.2	3.15	0.83

exhibit enhanced photo stability when compared with water-soluble dyes, the data in Table I indicate the reverse to be true for the tablets studied. For example, in the case of the red dye and lake, the k_1 at normal and exaggerated illumination for the lake is approximately five- and threefold that of the dye, respectively, while in the case of the blue dye and lake, the values are about seven and one and one-half-fold, respectively.

We have used initial fading rate constants as our criteria for evaluating the relative stabilities of the various colorants. It was felt that in so doing other contributing factors that may come into play during the later stages of degradation and have an effect on the fading rates are eliminated. However, in studying the data from this investigation, it may be interpreted, for practical purposes, that the overall long term stability of FD&C Blue No. 1 is poorer than D&C Blue No. 1 lake under normal illumination. This may be attributed to the fact that although the blue lake shows a more rapid initial degradation rate than the blue dye, its subsequent degradation is substantially slower than that of the dye, as illustrated by Figs. 3 and 4. For both yellow and red colorants, either method renders identical results, with the dye being superior.

The influence of particle size of the lakes on light stability has not been studied in this investigation. It is well known, however, that the particle size distribution of the lakes and pigments tend to affect light stability. Bean and Rowe (7) state that the fastness to light of materials dyed with a given insoluble azo color increases with a growth in the size of the pigment particles remaining in the fiber. The additional light stability is attributed to a decrease in surface area of the coloring matter exposed. Baxter, *et al.* (6), in studies involving textiles indicated that pigments of certain specific particle sizes exhibited enhanced light fastness when deposited on cellulosic fibers. Although no attempt was made to determine the particle size distribution of the lakes used, it is felt, however, that a study relating particle size to light stability would be interesting.

It has been observed previously that color fading takes place beneath the surface of tablets to varying extents. It was believed that tablets colored with lakes would show less depth fading than that found for the soluble dyes. This would be due to the insoluble nature of the lakes as well as the greater density of tablets prepared by the precompression method. This was not confirmed by a study of samples microtomed to various depths from the surface.

SUMMARY AND CONCLUSIONS

1. The relative light fastness of several certified water-soluble dyes and their corresponding lakes were investigated.

2. Initial fading rate constants were used as criteria for determining the light fastness of the colorants.

3. Lakes prepared from FD&C Red No. 1, FD&C Blue No. 1, and FD&C Yellow No. 5 exhibited less photostability than the dyes in the system studied.

4. Tablets employing lakes as colorants manufactured by the precompression method did not demonstrate greater resistance to depth fading than tablets containing dyes prepared by wet granulation techniques.

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Color Stability of Tablet Formulations IV

Protective Influence of Various Colored Glasses on the Fading of Tablets

By C. J. SWARTZ, L. LACHMAN, T. URBANYI, and J. COOPER

The protective influence of glass of various spectral transmission characteristics on the light-induced fading of colored tablets has been investigated. The glasses were evaluated as to their effect on the fading rate of tablets colored with FD&C Blue No. 1 and D&C Yellow No. 10. The glass samples evaluated included flint, Georgia green, emerald green, champagne green, and ultraviolet-absorbing green, as well as plastic coated lantern slides incorporating ultraviolet-absorbing agents. The apparent rates of fading of the dyes were determined following exposure of the tablets to exaggerated illumination. Reflectance measurements performed on the tablets at intervals indicated that amber glass apparently afforded the greatest protection against the fading of both dyes under the condition of study.

THE PROTECTION of dosage forms from the deleterious effects of light is no small problem in the pharmaceutical industry. Products exhibiting physical or chemical changes resulting from the effects of radiant energy can, in most instances, be adequately protected by the use of special glass containers. Flint glass, which is the most used multipurpose container material, has the disadvantage of being transparent to light rays above 300 mμ. As a result, amber colored glass and other special glasses which have the property of shutting out certain portions of the light spectrum have been used extensively by the pharmaceutical industry.

Though only limited work of a pharmaceutical nature has been published, a number of studies of the protective action of colored glasses have been performed on light-sensitive products in other fields such as the brewing, dairy, and food processing industries. A patent by Uihlein and Laurie (1) claims the stabilization of beer by the exclusion of radiation of wavelengths below 560 mμ with the use of red glass bottles. It has been shown (2) that deterioration in flavor and in certain constituents of milk often results from exposure to sunlight in flint glass bottles. McConnell and Esselen have shown that salad oils in flint containers develop rancidity on exposure to sunlight (3). These losses were minimized in amber bottles and even more so in ruby bottles which cut off practically all light shorter than 580 mμ.

With regard to pharmaceuticals, the U. S. P. has specified transmission characteristics for "light-resistant containers" for numerous medicinals in terms of the ultraviolet transmission of the glass. The pharmacopeial definition of a "light-resistant container" is based on specifications established from amber glass samples. These standards resulted from studies such as those performed by Arny, *et al.* (4), as well as Jermstad and Ostby (5), indicating that many products are deteriorated in containers permitting the passage of ultraviolet light, while amber glass was found to afford sufficient protection. Consequently, amber glass has become the only colored glass to achieve wide usage in containers for pharmaceutical products.

It was thought, however, that satisfactory protection of dosage forms could be achieved by glass containers with reduced light transmission characteristics other than those of amber glass. This report is concerned with a study of the fading at the surface of tablets protected from the radiation source by various colored glasses. The color stability was determined following exposure to so-called "exaggerated" light intensity which approximates fifteen times average room illumination using fluorescent light sources.

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EXPERIMENTAL

Materials Used.—FD&C Blue No. 1, Calco Chemical Co., sample 60803. D&C Yellow No. 10, H. Kohnstamm and Co., sample K7059. The several specially prepared glass samples used in this study were (*a*) bottle glass: flint, amber, Georgia green, emerald green, champagne green, and ultraviolet-absorbing green; (*b*) flint plate glass; (*c*) plastic coated lantern slides incorporating ultraviolet-absorbing agents. The glass samples used were either $3^1/_4$ inch square and 2 mm. thick or $3^1/_4 \times 4$ inches and 2 mm. thick.

Equipment.—An accelerated light stability cabinet described in a previous publication (6). Stokes model E tablet press. Beckman spectrophotometer model DU with reflectance attachment and die for centering and leveling single tablets, reported in an earlier paper of this series (7). Gossen Tri Lux foot candle light meter. Beckman Aquameter.

Preparation of Tablets.—Tablets colored with the above two dyes were prepared according to the following formula

Calcium sulfate dihydrate	96.99%
Tragacanth U. S. P.	2.00%
Magnesium stearate U. S. P.	1.00%
Color	0.01%
Ethyl alcohol 50%	q. s.

A granulation of the above formula was prepared by dissolving the dye in the 50% ethyl alcohol and then granulating the calcium sulfate dihydrate and tragacanth with this solution. The rest of the procedure followed commonly-employed tableting techniques. Tablets weighing 100 mg. were compressed using $^{5}/_{32}$-inch flat punches, uppers bisected. The tablets were compressed to a hardness of 6 Kg./in.^{2}, a thickness of 2.3 mm., and with the moisture content of the dried granulation below 0.5%.

Storage of Tablets Under Glass Samples of Different Spectral Transmission.—The influence of these glasses on the fading of tablets colored with FD&C Blue No. 1 and D&C Yellow No. 10 exposed to a lighting system of high intensity was studied. Tablet samples were prepared for testing in the exaggerated lighting cabinet by placing adequate samples into aluminum dishes 76 × 10 mm. in size. These dishes were, in turn, placed under glass sleeves measuring $3^1/_4 \times 1^1/_4$ inches. The several glasses to be evaluated were cemented to the tops of the sleeves. The walls of the sleeves were covered with masking tape to prevent light penetration from the sides. The sample dishes were then placed into the accelerated light cabinet. In addition to these dishes, control samples of blue and yellow tablets were placed into the cabinet without any glass cover over the sleeves. The intensity of illumination in this cabinet was about 550 foot candles. Samples were withdrawn from the various dishes at regular time intervals for reflectance measurements. Samples of blue and yellow tablets were evaluated with the several bottle glass and flint plate glass disks, while only the blue tablets were evaluated against the glasses coated with ultraviolet absorbers.

Measurement of Tablet Reflectance.—The procedure and equipment employed to follow changes in the reflectance of the surface of the tablets were described in previous publications (7, 8). Reflectance measurements were taken at 640 mμ for FD&C Blue No. 1 and 420 mμ for D&C Yellow No. 10.

Preparation of Glass Samples to Be Used for This Study.—In the case of bottle glass, suitable pieces were ground and polished so that their surfaces were reasonably flat and parallel. An effort was made to finish the samples to an approximate thickness of 2 mm. Transmission measurements were made with reference to air using a General Electric recording spectrophotometer and a Beckman model DU spectrophotometer. From the data as measured, transmission curves of the glasses to be evaluated are presented in Figs. 1 and 2 (9).

RESULTS AND DISCUSSION

In order to evaluate the influence of the several glass samples on the fading properties of the tablets colored with FD&C Blue No. 1 and D&C Yellow No. 10 the spectral absorbance at the surface of the tablets was measured at prescribed time intervals.

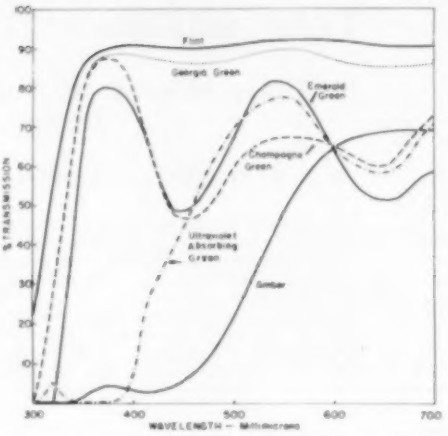

Fig. 1.—Light transmission curves of various colored glasses.

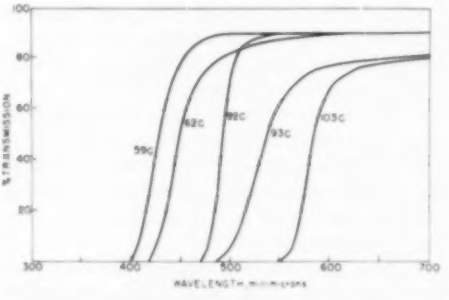

Fig. 2.—Light transmission curves of glass samples coated with plastic films of ultraviolet light absorbers.

A representative plot presented in Fig. 3 shows the influence of glass sample 92C on the absorption from the surface of tablets colored with FD&C Blue No. 1. It is evident from the spectral absorbance curves in this figure that glass sample 92C (containing a plastic film of ultraviolet absorber) exerts an appreciable retarding effect on the color fading of the tablets when compared with the control sample in an uncovered dish. The nature of the change in the curves with time were similar for glass samples 93C and 103C which also contained a film of ultraviolet absorber on their surfaces. As was found in our previous studies with FD&C Blue No. 1 (8, 10), the spectral absorbance curves obtained in this study showed a hypsochromic shift and a decrease in height of the absorption maximum as fading progressed. The protective effect of these three glass samples containing films of ultraviolet absorbers seemed to be dependent on the wavelength of light where transmission of radiant energy began. This point will be discussed in greater detail in a subsequent section of the report when fading rates are correlated with the transmission characteristics of the glasses.

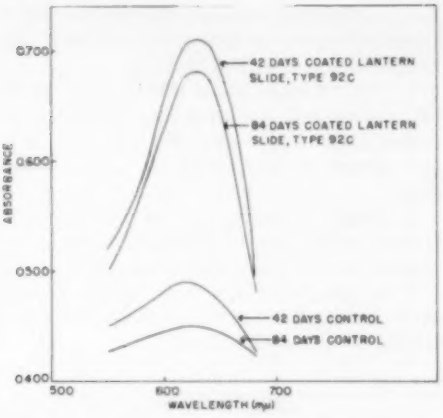

Fig. 3.—Plots of the visible absorption spectra of FD&C Blue No. 1 obtained from tablets protected by coated lantern slides with U. V. absorber.

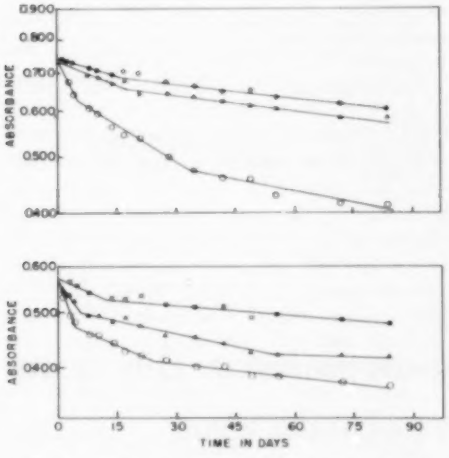

Fig. 4.—The protective effect of amber and ultrasorb green glass samples on the fading of the surfaces of tablets colored with FD&C Blue No. 1 (top graph) and D&C Yellow No. 10 (bottom graph). ○, Control; □, amber glass; ▲, ultraviolet absorbing green.

To obtain a comparative picture regarding the color fastness of the yellow and blue tablets exposed to exaggerated illumination in dishes covered with various glass samples, the fading rates were calculated for these tablets following storage. These fading rates were obtained from plots as represented in Fig. 4. Since these curves show a linear regression of absorbance with time, the fading seems to follow an apparent first-order reaction. In addition, as was evidenced in our previous work, the graphs show more than one slope. Since it has been pointed out before that the rate constants obtained from the first slope of the curve most accurately represent the photodegradation of the dye at the tablet surface, only the initial rate constants will be used in our subsequent discussions. The rate constants evaluated from the initial slopes of the lines of log absorbance *vs.* time are presented in Table I.

Table I.—Rate Constants in Days^{-1} for the Fading of Dyes Irradiated Through Various Glass Samples

Glass Sample	FD&C Blue No. 1 $K_1 \times 10^2$	D&C Yellow No. 10 $K_1 \times 10^2$
Control direct exposure	17.9	39.7
Flint glass	12.9	38.1
Regular flint plate	13.0	37.8
Georgia green	13.2	37.4
Emerald green	11.4	28.6
Champagne green	11.2	26.2
Coated lantern slides 59C	9.3	...
Ultraviolet-absorbing green	5.5	18.1
Coated lantern slides 62C	5.1	...
Amber	4.6	6.2

It is evident from this table that for the tablets colored with FD&C Blue No. 1, amber glass gives the best protection of the glasses tested. Slightly less protective than amber are ultraviolet film-coated glass sample 62C and ultraviolet-absorbing green followed by ultraviolet film-coated glass sample 59C. Next in the series are champagne and emerald green, with Georgia green, regular flint plate, and flint glass closely behind. From the transmission curves shown in Fig. 2 it is possible to explain the enhanced stabilizing effect of glass sample 62C over 59C. The additional low wavelength radiation which is transmitted through glass sample 59C is most likely the causative agent for the increased fading of the blue color from the surface of the tablets. The transmission curves for the colored glass samples presented in Fig. 1 can be used to explain the fading rate constants given in Table I. Because of the absorption characteristics of the amber glass it would be expected to show the greatest protective action against fading when compared with the other glass samples. The other glasses in Fig. 1 follow a similar pattern depending upon their transmission properties.

In observing the data for tablets colored with D&C Yellow No. 10 and stored similar to the blue tablets using the glass samples shown in Fig. 1, a like pattern was noted relative to the fading rates and transmission curves. Ultraviolet film-glass samples were not used with the yellow tablets because sufficient samples of glass were not available.

It will be noted from Table I that the rate constants for the fading of D&C Yellow No. 10 colored tablets are at least twofold that of the FD&C Blue No. 1, with the exception of those tablets exposed under amber glass. This may possibly be ascribed to the fact that D&C Yellow No. 10 absorbs strongly between 410–430 mμ, in which region all the glasses tested, except amber, transmit significantly. On the other hand, because FD&C Blue No. 1 absorbs strongly in the range of 610–650 mμ, tablets colored with this dye would be substantially less affected by the light of lower wavelength transmitted

through the several glass samples. In the case of amber glass, the fading rates of both colorants at the surface of the tablets are rather similar.

Since the intensity of the illumination and the spectral character of radiation generally influence fading, results obtained in this study might be modified if other sources of radiation were employed.

It should be remembered that it has long been recognized that energy must be absorbed in order to be effective in fading colored materials. Therefore, we might expect a yellow colored tablet to fade faster under lower wavelength radiation than a blue colored tablet because of the absorption characteristics of these two colors. However, it has been reported that the absorption of energy at a particular wavelength does not necessarily result in fading, and therefore, the color of the material may not give any indication of the wavelength of radiation which will cause the greatest amount of fading. In fact, the results of Luckiesh and Taylor (11) show that there is no conspicious relationship between the wavelength of the energy absorbed (in the visible spectrum) and the amount of fading produced. Hence, the absorption spectrum of most colored materials cannot do more than indicate a band of radiation among which those responsible for fading are to be found.

SUMMARY AND CONCLUSIONS

The influence of glasses with various light transmission characteristics on the fading of colored tablets has been investigated and a summary of the results follows:

1. Amber glass afforded the best protection against fading for the tablets colored with FD&C Blue No. 1 and D&C Yellow No. 10.

2. For the blue colored tablets, glass sample 62C coated with an ultraviolet absorber, and colored glass sample ultraviolet-absorbing green gave almost as good protection as the amber glass.

3. Glass containing a film of ultraviolet absorber appears to show promise for use in the prevention of photodegradative reactions.

4. A correlation has been found to exist between fading rates of the colors and light transmission of the glasses studied.

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Stability Studies of Dihydrostreptomycin Ointments

By FRANK P. COSGROVE and CHARLES F. POE

Dihydrostreptomycin was used as the active ingredient in polyethylene glycol-type bases and in petrolatum base ointments. The different ointments were analyzed over a period of five years for loss in antibiotic activity. The polyethylene glycol ointments, when stored at 5°, lost less than ten per cent activity in two years, and 20 per cent or less in five years. The activity lost, when stored at 25°, was somewhat greater. The petrolatum base for dihydrostreptomycin was not satisfactory after long storage.

VARIOUS COMPARISONS have been made on the activity of antibiotics in different ointment bases. However, very little information is available on the activity of dihydrostreptomycin in ointments during storage at different temperatures. The purpose of this study was to determine the stability and activity of dihydrostreptomycin in various polyethylene glycol-type ointment bases stored at 5 and 25° for a period of five years. For comparative purposes, a petrolatum base ointment also was tested.

The petrolatum or nonemulsified type of ointment base now used for the official penicillin ointment has been shown to possess many disadvantages. Fantus (1) and Goodman (2) stated that such bases interfere with many of the normal skin functions such as heat control and discharge of perspiration. These conditions also tend to make the skin impermeable to the medi-

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TABLE I.—RETAINED POTENCY OF DIHYDROSTREPTOMYCIN IN VARIOUS OINTMENT BASES DURING STORAGE

Storage Temp., °C.	Initial Zone of Inhibition, mm.	Storage Time, Months										
		1	3	6	12	18	24	30	34	36	48	60
					Base A							
5	30	100[a]	100	93	86	80	77	76	72	70	68	64
25		100	97	89	79	75	70	67	61	59	57	55
					Base B							
5	38	100	100	98	98	95	93	90	85	82	80	80
25		100	98	93	90	85	83	80	78	75	74	74
					Base C							
5	40	100	100	98	96	93	92	90	84	83	82	80
25		100	98	94	91	86	81	80	73	73	72	72
					Base D							
5	37	100	100	100	97	95	92	90	87	84	84	83
25		100	97	95	92	88	84	81	76	76	75	75

[a] Percentage of the zone of inhibition remaining in comparison with that of the ointments when first prepared; the results are an average of the three determinations.

cinal agents present in the base. In addition to these disadvantages, Munford (3) explained that much of the cooperation required by the patient in carrying out routine application of the ointment is lost when these bases are used because such ointments soil clothing and bed linen and because they are difficult to remove from the body.

Considerable work has been done on the penetration of sulfonamides through intact skin from various ointment bases. Woodward and associates (4) studied the penetration of sulfonamides into intact rabbit skin by direct chemical analysis of skin biopsies, as well as by blood and urine analysis. The effects of time of applications, concentration of sulfonamide, vehicle, and surface-active agents were studied. The usual water-in-oil, oil-in-water, or jelly bases as sulfonamide solvents were inferior to glycol bases. Clark (5) reported that polyethylene glycol compounds are ideal for sulfonamide ointments because of low toxicity, greater solvent power, and high degree of penetration of the drugs through the skin.

On the other hand, the petrolatum bases are more satisfactory for ointments containing certain antibiotics, especially penicillin and bacitracin. Culter (6) noted that a greater degree of penicillin decomposition occurred in the polyethylene glycol ointments than in the anhydrous petrolatum bases. Penicillin in polyethylene glycol bases lost all potency in a little over two weeks when stored at room temperature and 81 per cent of potency when stored in a refrigerator for one month. Sherwood and Mattocks (7) found that an ointment containing polyethylene glycol 1000 and polyethylene glycol 600 inactivated penicillin rapidly.

Florestano, Bahler, and Jeffries (8) reported that bacitracin, polymyxin, and neomycin were more stable in, and more readily released from, bases containing ethylene glycol diesters than with the grease-type bases. Lockie and Sprowls (9) reported that the diffusion rates of certain drugs in polyethylene glycol ointment were much higher than those from grease or water-in-oil bases. Plein and Plein (10) tested the action of antibacterial drugs in several silicone bases and petrolatum bases. It could not be shown that there was any difference in efficiency of either class of bases when chlortetracycline was added.

METHODS AND PROCEDURES

In the preparation of the ointment bases, each ingredient was dried at a temperature of 125° for a period of one hour. For experimental purposes, four different formulas were chosen, as given below:

Base A

Wool fat	20.0%
Liquid petrolatum	25.0%
White petrolatum	55.0%

Base B

Polyethylene glycol 1500[1]	100.0%

Base C

Polyethylene glycol 4000[2]	45.0%
Propylene glycol	55.0%

Base D

Polyethylene glycol 1500[1]	36.0%
Polyethylene glycol 4000[2]	20.0%
Cetyl alcohol	4.0%
Propylene glycol	40.0%

The weighed amount of antibiotic was added to the melted base which was cooled to about 50°. The mixture was stirred or levigated lightly until a smooth ointment resulted. The preparations were immediately placed in a collapsible tube. Each

[1] Carbowax 1500, Carbide and Carbon Chemical Corp.
[2] Carbowax 4000, Carbide and Carbon Chemical Corp.

ointment contained 5,000 mcg. of dihydrostreptomycin.

To test the antiseptic value of the ointments, the FDA agar-cup-plate method (11) and the Vincent disk method (12) were used. The test organisms were *Staphylococcus aureus* and *Micrococcus flavus*. Ten per cent horse serum was added to the Penassay base and seed agars since the ointments tested might be used on open wounds, cuts, or in the presence of serous fluids.

Enough ointment from the tube was used to fill exactly the cup in the agar or cover the disk. The plates were incubated for eighteen hours at 37° and the zones of inhibition were measured. The loss in activity was determined by comparison of the diameter of the zone produced at a given time with that of the zone produced by the ointment when first prepared.

EXPERIMENTAL RESULTS

Each ointment was tested immediately after preparation and samples were stored at 5 and 25°. These samples were analyzed in triplicate at intervals of seven, fourteen, twenty-one, and thirty days, and then every two or three months until the end of five years. The results with *Staphylococcus aureus* are listed in Table I for only some of the representative periods. The results are expressed as percentage of potency retained during storage with time.

Controls conducted on the base ointments without antibiotics showed little or no evidence of antibacterial activity. The petrolatum greasy type base A showed no action whatsoever. The polyethylene glycol-type ointments containing the antibiotic exhibit greater penetrating power than the greasy type ointment.

All of the ointments containing the polyethylene glycol compounds and stored at 5° showed excellent keeping qualities during the first two years. The loss of activity the first two years was less than 10%, and during the third year around 17%. At the end of five years the loss of activity was 20% or less. The losses of activity at the storage temperature of 25° were much greater than those resulting in the ointments stored at 5°. The activity of the dihydrostreptomycin in the greasy type ointment held up very well for six months, but after a year of storage at both temperatures the activity decreased fairly rapidly.

The results obtained with *Micrococcus flavus* are not reported in this publication, but the same order of effects were noted as with *Staphylococcus aureus*, except that the zones of inhibition, in general, were somewhat larger with *M. flavus*.

CONCLUSIONS

1. Usually the polyethylene glycol-type ointments containing the dihydrostreptomycin exhibited more diffusion of the antibiotic throughout the test medium than the petrolatum-type ointment.

2. The zones of inhibition were greater in the polyethylene glycol ointment than in the petrolatum-type ointment.

3. The dihydrostreptomycin in the polyethylene glycol ointment retained activity during the first two years when stored at 5°. The losses at the end of five years were 20 per cent or less. The activity of ointments stored at 25° decreased more rapidly, but losses in eighteen months were only around 15 per cent.

4. After the first year, the petrolatum-type ointment showed a fairly rapid loss in activity at both temperatures.

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Equipment and Methodology for Relating Gastrointestinal Absorption to Site of Drug Release

By S. P. ERIKSEN†, J. V. SWINTOSKY, E. J. SERFASS‡, T. H. LIN, J. ABRAMS, and F. M. STURTEVANT§

A radio frequency generator operating at 500 kilocycles with a 2½ kilowatt maximum power output has been constructed and used for opening specially designed capsules at selected sites in the gastrointestinal tract without direct connection of the capsule to equipment outside the body. The procedure is particularly adapted to the study of gastrointestinal absorption as a function of site of drug release under normal physiologic conditions. The equipment and technique have been utilized in studies of salicylate absorption in dogs and appear applicable to absorption studies with human subjects.

THIS REPORT describes the design, construction, and use of a radio frequency generator suitable for opening a specially designed capsule and releasing a drug at selected sites within the gastrointestinal tract without surgery or direct attachment of the capsule to accessory equipment outside the body of the test subject. The equipment and technique have been used to study the absorption of salicylate as a function of site of drug release in dogs.

The rates, efficiencies, and mechanisms of absorption of drugs and foodstuffs from the gastrointestinal tract have been of considerable interest for many years. Pharmaceutical investigators have been particularly concerned with the effect of drug release site on the rate and efficiency of drug absorption because of the bearing such effects have upon design and evaluation of oral dosage forms.

Available methods for studying absorption include the gastrointestinal fistula and the Miller-Abbott tube, or variations thereof. While useful, these methods create abnormal conditions in the subject. The results are complicated by the unknown effects of these abnormal conditions upon absorption. In addition, these methods are not sufficiently convenient for routine use on a large scale.

It was against this background that we developed an external radio generator that would open a drug-containing capsule in the gastrointestinal tract. Since our work started, a report appeared that described the use of radio frequency induction heating in connection with a mechanical capsule for removing samples from the gastrointestinal tract (1). To our knowledge, the release of a drug by use of a radio frequency generator and the description of suitable equipment have not been reported previously.

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EXPERIMENTAL

Electrical Equipment.—A Lepel[1] high frequency bombarder unit, model T 2½ 1B has been modified for use in our device. The circuit diagram of this radio frequency generator is given in Fig. 1. Briefly, it is a modified Hartley oscillator circuit (2a) utilizing a Lepel F-6399 oscillator tube. The 220 volt a. c. input is increased to approximately 3,000 volts in the power transformer before full-wave thyratron rectification. The unfiltered d. c. signal is applied to the plate circuit of the oscillator tube through a phase-shifting network used to control the power output. The tank coil of the oscillator is divided into two parts, the major portion being inductively linked to the coil in the grid feedback circuit to produce oscillations of approximately 470 kc., varying slightly with the load in the output coil. The remainder of the tank coil is led outside the generator to the output coil, where the usable electromagnetic field is created. The output from the rectifiers and phase shifter is about 3,000 volts at 0.7–1 ampere, giving a nominal power rating of 2½ kilowatts for the generator.

Two interchangeable, water-cooled output coils have been made, one 14 inches i. d. (Fig. 2) and one 5 inches i. d. The larger coil is made of six turns of ¼ inch i. d. copper tubing wound around a 14-inch Bakelite core. The smaller coil consists of twelve turns around a 5-inch core. The outsides of the coils are enclosed to prevent electrical shock.

The operation and relative field strength of the generator and coil were monitored with a Heathkit relative field strength meter (model PM-1) placed in a standard position near the coil and subject.

[1] Lepel High Frequency Laboratories, New York, N. Y.

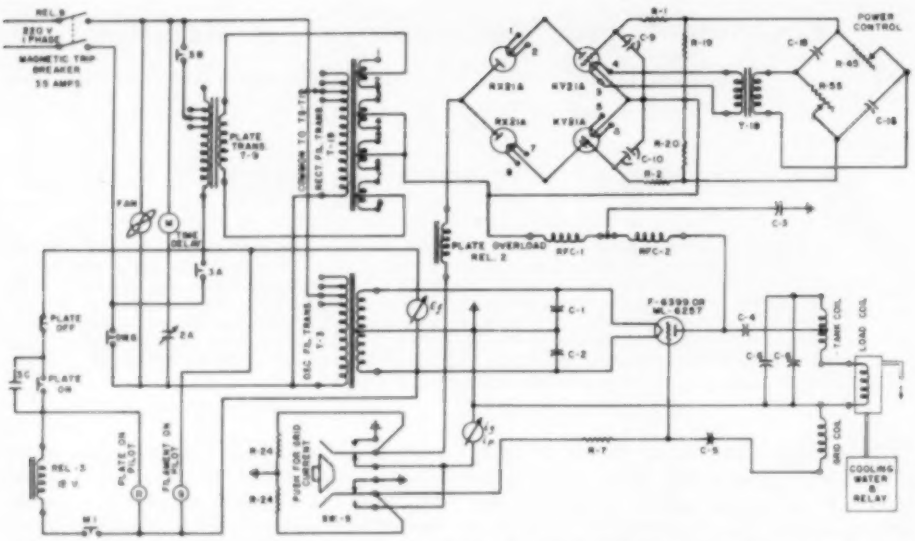

Fig. 1.—Radio frequency generator. Except for the load coil and cooling water relay and safety switch the circuit is essentially that of a Lepel high frequency bombarder, model T 2½ 1B. The cooling water relay and the safety switch (SW-6) is set for 1.25 gallons per minute; the load coils are specially made as described in the text. The complete assembly was built by Serfass Corp., 345 Conestoga St., Bethlehem, Pa.

Capsule Construction.—The basic capsule design for use in this device is shown in Fig. 3. The inside metal sleeve (Fig. 3 *B*) is made of seamless Inconel[1] tubing, a stainless steel of low magnetic susceptibility. The metal tubes have dimensions of 0.312 inch o. d. × 0.020 inch wall thickness × 0.625 inch long. When the position of the capsule in the intestine is followed with thread, a tiny brass ring is soldered to one end of the tube; otherwise, this ring is omitted. The metal tube (and ring) is covered with a thin (0.02-inch wall) polyethylene sleeve (Fig. 3 *C*).

Inside the capsule is a spring and pusher assembly. The springs (Fig. 3 *E*) are constructed of six turns of 0.007 inch diameter music wire, with an overall length of $^{5}/_{8}$ inch. The pusher (Fig. 3 *D*) is constructed of a $^{1}/_{16}$ inch strip of 22 gauge (0.031 inch) galvanized iron, bent to the shape shown. A piece of $^{1}/_{8}$ inch polyethylene tubing is slipped over it and the pusher is soldered to the spring. The opposite end of the spring is soldered to the rear edge of the Inconel sleeve (Fig. 3 *F*).

When tablets are used, they are prepared with $^{3}/_{16}$ inch flat face punches. One large or several small tablets may be used. For liquids or powder, a 0.20-inch diameter tempered brass wire frame (Fig. 3 *A*) is inserted on top of the pusher. This frame is bent to the shape shown and all joints are soft-soldered.

To seal the capsule, the spring is kept depressed with a small bit of wire through the back of the pusher and the whole capsule is dipped into melted wax[2] at 80–90° and held until all spaces are filled.

Fig. 2.—The exterior of the large induction heating coil, showing dog in place while the capsule within its gastrointestinal tract is being opened.

When the wax has solidified, the wire is removed, the back sealed by dipping, and the protruding end of the polyethylene on the pusher is exposed with a sharp knife (Fig. 4 *A*).

To fill with a liquid, a frame is first dipped in wax and then dropped into the wax-coated tube described above. The front of the capsule is then

[1] Trademark of the International Nickel Co. for its nickel, chromium, and iron alloy.

[2] A homogenous mixture of Fisher's Tissue mat (m. p. 53°) and microcrystalline wax (m. p. 77–80°), 50% w/w of each.

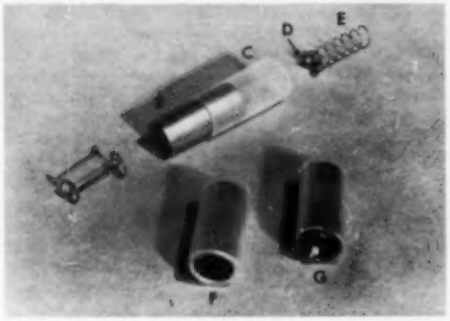

Fig. 3.—Various stages in the manufacture of capsules for use in the radio frequency generator. *A-E*, an exploded view of capsule and parts, shown assembled in *G* (front view) and *F* (rear view).

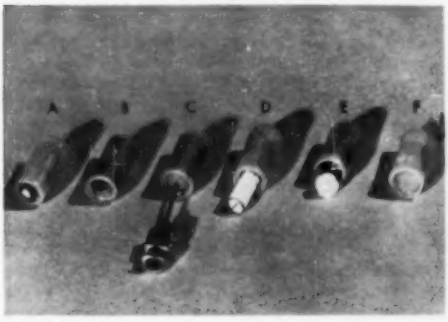

Fig. 4.—Various stages in the filling and sealing of capsules for use in the radio frequency generator. In *A*, the spring has been depressed, the capsule surfaces coated inside and out, the rear end sealed, and the polyethylene sleeve insulation on the pusher is exposed. *B* shows frame in place. In *C* the frame has been sealed within the capsule and the capsule is being filled with drug solution. *D* and *E* show compressed powder and tablet being placed in the capsule. *F* shows complete sealed capsule with a thread attached.

sealed with wax, using a hot spatula, after which the capsule is filled with a hypodermic needle (Fig. 4 *C*). Finally the hole created by the needle is sealed with a hot spatula. When tablets are used, they are dropped into the wax-coated tube described above (Fig. 4 *E*) and the front end is sealed. To fill with a powder, a frame is first placed in the base of a No. 3 gelatin capsule; powder is poured in and packed tight. The frame and compacted powder may now be pulled from the capsule base intact and sealed into the wax capsule as with tablets (Fig. 4 *D*). After final sealing, the excess wax is removed from the front end of the capsule in such a way that the front wax plug seals only the metal tube (see cross section, Fig. 5). The finished capsule holds about 0.5 ml. of liquid or 500 mg. of solid and has overall dimensions of about 0.38 × 0.81 inches.

Toxicity Studies.—Sixteen Dierolf rats, averaging 55 Gm. body weight, were placed in plastic containers two hours per day each weekday for two

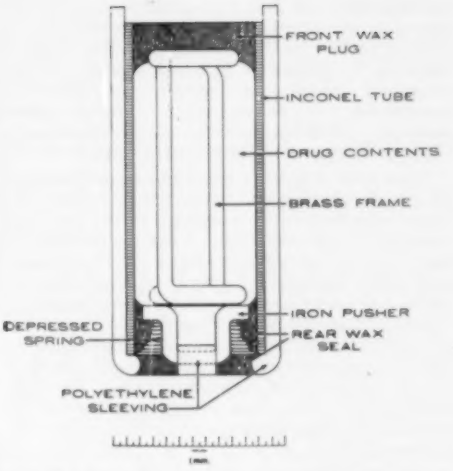

Fig. 5.—Scale drawing of cross section of sealed capsule.

weeks. Eight of the rats were placed in the center of the field of the 5-inch output coil of the generator during this period and the reminder (controls) were placed outside of the field. The power applied was approximately 2.1 kilowatts at 470 kc. Following this course of treatment, the rats were sacrificed with chloroform, autopsied, and the adrenals, hearts, kidneys, testes, and thymuses weighed. The adrenals, kidneys, and duodena were examined for histological changes.

Salicylate Absorption Studies.—Capsules for use in salicylate absorption studies were filled with 0.4 ml. (275 mg.) of a 68.1% w/v solution of sodium salicylate in distilled water.

The capsules were administered to trained, fasted (eighteen hours) dogs and sufficient time was allowed for the capsule to reach the intestinal area desired, either as measured on an attached thread or estimated fluoroscopically.

The drug was released by placing the dog in the coil of the generator for approximately three minutes, after which fluoroscopy was employed to establish that the capsule was opened and the drug had been ejected. Blood samples taken at one-fourth, one-half, one, two, three, four, six, and eight hours were analyzed for salicylate content using a ferric chloride colorimetric method (3) and the data plotted as mg./100 ml. plasma *versus* hours after drug release.

RESULTS AND DISCUSSION

Radio Frequency Generator.—The radio frequency generator described is, in essence, a low power induction heating device. While the theory of induction heating is well-documented (2*b*), a brief discussion of the principles and their utilization in this device may be beneficial. In operation, the generator produces an alternating current throughout the external load coil, which, in turn, produces an alternately increasing and decreasing magnetic field around and inside the coil. This

changing field can induce three different effects on materials exposed to it: (*a*) Eddy currents may be induced to flow in a conducting material. In this instance, the conductor functions similarly to the secondary coil of a transformer. (*b*) Magnetic polarization may be induced in a ferromagnetic material. (*c*) Orientation of molecular dipoles may occur in a dielectric (nonconductive) material.

From each of these induced effects, heating may occur. In nonmagnetic conductors, the eddy currents cause heating in proportion to the electrical resistance of the conductor. When the resistance is low (e. g., in brass, silver, and gold), the heat generated will be negligible. In magnetic conductors, the additional energy absorbed as a result of the rapidly reversing magnetic polarization can cause considerably greater amounts of heat. Heat is generated in dielectric materials as a result of the energy absorbed by the orientation of molecular dipoles. The heat produced is related to the dielectric constant of the material, the field strength, and the frequency of the alternating field.

Frequency influences the heating efficiency in each of these cases. In conductors, low frequencies (10–1,000 kc.) are quite efficient, while in dielectric materials, frequencies greater than 1 mc. are required for efficient heating. Dielectric materials such as tissue require at least 10 mc. for efficient heating by radio frequency waves (diathermy) (4).

For our work, it was desirable to construct a generator with power and frequency characteristics that would selectively heat a metal capsule, while having no effect on the surrounding tissues. Frequencies of about 500 kc. seemed well-suited for the differential heating required, as these frequencies have only a slight diathermic effect and yet are very effective in heating metals. Using Inconel for the metal in the capsule insures slow heating by eddy current loss, and this obviates the need for accurate timing of irradiation.

Capsule Design and Operation.—The capsule itself was designed to be simple, to withstand the peristaltic action of the intestinal tract without opening prematurely, to require relatively low temperatures for complete opening, and to be adaptable to many drug forms. The wax used is indigestible and melts at a temperature which is sufficiently high so that it is hard at body temperature; capsules have been opened after being in the dog's intestinal tract for twelve hours.

When the capsule is placed in the field of the generator, the metal parts begin to warm. The warming pusher frees the spring and the warming tube frees the plugs. At about 50–52° the spring releases, pushing the frame (or tablets) forward with the front wax plug. The pusher itself, moving forward, pulls a hole in the rear wax seal allowing the contents to drain when liquid is present in the capsule. The pusher stops about $^1/_8$ inch short of the front opening of the capsule so that the metal portions of the capsule are retained within the polyethylene sleeve and cannot touch any portion of the gastrointestinal tract (the pusher has been pulled out in Fig. 3 *G* so that it can be seen). One can be certain the capsule has opened when a fluoroscopic observation indicates that the frame is outside the capsule.

Despite the high power available from the generator, the capsule absorbs relatively small amounts of energy. Using thermocouple temperature measurements, an empty capsule hanging in air inside the coil absorbs about 0.9 watts, calculated on the basis of the rate of temperature rise of the 2.5-Gm. metal sleeve. In the dog, however, the same capsule absorbs about 0.2 watts. This shielding by the dog's body (approximately 80% decrease, due to signal attenuation and heat dissipation) seems to be fairly constant for dogs of 11–16 Kg. body weight.

The heat generated by the capsule during opening may have some effect upon absorption, although at the temperatures involved this would be small.

Position of the Capsule in the Gut.—Because it was our intent to study absorption as a function of site of drug release (i. e., with respect to various sections of the gastrointestinal tract), rather than at the site of the capsule, the exact position of the capsule is not very critical. This is because absorption occurs as the drug moves along the tract after release; with slowly absorbed drugs this may occur over a considerable area of the intestinal tract.

In some of the early studies described in this report, the distance down the tract was measured with a trailing length of Dacron sewing thread marked off in feet. In these studies it was found that although the dog swallowed extra thread during the experiment, this extra thread could be retrieved by pulling gently, thus assuring an accurate measurement of capsule position in the tract. Fluoroscopic observations were later substituted for the thread measurements. By comparison with thread measurements, the accuracy of the fluoroscopic distance estimates was found to decrease as the capsule passed down the gastrointestinal tract. Within one foot of the pylorus, estimates within two or three inches could be made, while in the ileal region the precision was one to two feet. The accuracy of the fluoroscopic method in the distal portions of the gut was found using dogs whose gastrointestinal tract had been marked in one-foot intervals with tiny, radio-opaque silver rings sewed to the intestine. Dogs prepared in this way can be used to train fluoroscopists for distance estimation, or for actual use in absorption studies. Silver rings do not warm sufficiently in the radio field to cause harm or absorption changes.

Safety and Toxicity Studies.—No conveniently accessible reports on the toxicity of radio frequency power have appeared in the literature but the considerable exposure to radio frequency energy that has been tolerated safely for years by men working in close proximity to commercial high power radio broadcasting equipment and induction furnaces suggests that such toxicity is low, if present at all. In the brief studies described in this report the rats subjected to repeated exposure of 2 kilowatts of 500 kc. power showed no gross or microscopic evidence of toxicity (Table I).

When the capsule temperatures were measured in dogs, the warmest part of the capsule was found to be the pusher (58–59°); the other metal parts heated to about 50–53° during the three-minute treatment. The solution within the capsule warmed to about 52–53°. The parts of the gut or its contents just adjacent to the capsule never rose above 52°. When irradiation was stopped, temperatures returned to normal in two to five minutes. We felt safe, therefore, in assuming that the radio frequency

TABLE I.—WEIGHT COMPARISONS OF CONTROL RATS *Versus* THOSE SUBJECTED TO 470 kc. RADIO FREQUENCY ENERGY

Group	No. Rats	Body Weight, Gm.		Organ Weight, Gm.				
		Initial	Change	Adrenals	Heart	Kidneys	Testes	Thymus
Treated Rats[a]	7	54.0	+61.9	0.031	0.52	1.28	1.32	0.35
Controls	7	54.7	+60.6	0.029	0.48	1.24	1.44[b]	0.28

[a] Using the rank-sum test (5), there were no significant differences (at $P = 0.05$) between growth rate, body weight, or organ weight of the treated and control groups.
[b] 6 Rats (1 female).

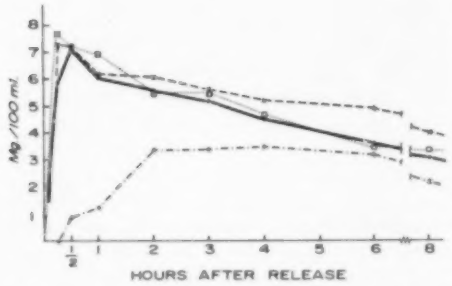

Fig. 6.—Salicylate blood level as a function of time, following release at several sites in gastrointestinal tract of a dog (mongrel, female, 16 Kg., dose 275 mg.). —— x —— x–, released in stomach; ...□...□..., released 12–15 inches past pylorus; —●—●—, released 26 inches past pylorus; ●—·—·●—·—, released in cecum.

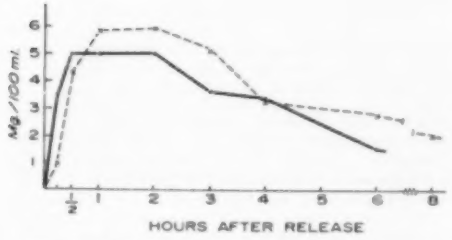

Fig. 7.—Salicylate blood level as a function of time following release at several sites in gastrointestinal tract of a dog (mongrel, male, 11 Kg., dose 275 mg.). —x——x——x—, released in stomach; —●—●—, released 30 inches past pylorus.

power itself would probably not alter the absorption characteristics of the intestinal tract.

Gastrointestinal Absorption.—The results of a study of salicylate blood concentration in two dogs as a function of release position in the gastrointestinal tract are shown in Figs. 6 and 7. For comparison purposes, we may consider two features of these curves, i. e., rate of absorption and efficiency of absorption. In Fig. 6, the absorption rate as approximated by the time to attain the peak blood level, and the absorption efficiency as approximated by the peak blood level attained along with the areas under the curves, are independent of release site from the stomach to two to three feet past the pylorus. Absorption in this region is essentially complete in fifteen to thirty minutes. This suggests then that the absorption characteristics of salicylate are the same over at least the first half of the small intestine.

In the cecum a different picture is observed; the rate and efficiency of absorption are both found to be less than in the small intestine. Absorption continues for two to three hours after release in the cecum, the peak blood level reached is less than half that observed in the intestine, and the area under the curve is also considerably smaller. In Fig. 7, data are shown for an experiment on another dog. Again, the rate and efficiency of absorption are independent of site of release from the stomach to two to three feet past the pylorus.

The picture drawn from this brief study is one of rather uniform absorption of salicylate throughout the stomach and upper small intestine. Also, an appreciable capability for absorption occurs even when the drug is released as far down the tract as the cecum. It should be remembered that the data obtained with this device do not depict the absorption characteristics of any one gut section alone, but rather those of the section extending from the point of release to the point where absorption ceases. This complicates any comparison of these data to studies on isolated gut sections.

This approach to obtaining absorption data, of the type illustrated here with salicylate, is important for the design of dosage forms, especially those employing some form of delayed availability. It enables the formulator to determine if some advantage related to absorption may accrue from releasing a drug at a specific site when released (*a*) as such, (*b*) in some physically or chemically modified form, or (*c*) in the presence of specific additives. Good control exists for determining the site at which the drug is to be released. A further advantage results because only a single dosage unit needs to be prepared for any single test. This circumvents the usual formulation work of preparing large numbers of dosage units, development of suitable coating techniques, overcoming variability between dosage units, and such related problems that have usually confronted the formulator prior to testing a hypothesis in absorption. This approach permits the hypothesis to be proved or disproved before the laborious formulation work is necessary.

It is felt that this methodology will provide absorption data of greater pharmaceutical relevance than the isolated gut technique because of the ability of the drug to move freely through the tract after release, simulating the action expected from usual oral dosage forms. Furthermore, it appears that with proper safeguards such studies may be conducted routinely without danger and discomfort, using laboratory animals or human subjects. The availability of this research methodology to clinical

investigators would simplify and hasten the gathering of knowledge of the factors which influence gastrointestinal drug absorption in human beings.

SUMMARY

1. A radio frequency generator suitable for opening specially-designed capsules and releasing drugs at selected sites within the gastrointestinal tract of dogs has been described in detail.

2. This generator and capsule permit studies to be made of the effect of site of release on absorption rate and efficiency of orally-administered drugs.

3. A study of the effect of site of release of salicylate ion upon its rate and efficiency of absorption has been described as an example of the capabilities of this device.

4. Safety and simplicity of this methodology suggest its utility for studying factors influencing absorption from the gastrointestinal tract of laboratory test animals and human beings.

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Antagonism of Apomorphine by Chlorinated Phenothiazines

By A. M. BURKMAN

Induction of the pecking syndrome with apomorphine was attempted in pigeons premedicated with a variety of central nervous system depressants and ataraxics. Of the agents employed, the chlorinated phenothiazines (chlorpromazine, prochlorperazine, and perphenazine) proved most effective in inhibiting the apomorphine-induced response. Hydroxyzine exhibited a lesser but significant degree of inhibition while pentobarbital, morphine, nalorphine, promazine, mepazine, and meprobamate were ineffective in modifying the syndrome. Several of the premedicants (nalorphine, meprobamate, mepazine, and hydroxyzine) were observed to potentiate the ordinarily trivial emesis provoked in pigeons by apomorphine.

THE APOMORPHINE response in pigeons previously described and quantitated (1, 2) has been of considerable interest in this laboratory not only because it provides raw material for our continued studies in avian pharmacology but more especially because of the unusual hallucinotropic nature of this response.

Hallucinogens and psychomimetic agents such as lysergic acid diethylamide (LSD) and mescaline are known to evoke responses in laboratory animals (3, 4) and humans (5, 6) reflected in altered behavioral and encephalographic patterns that appear to simulate, at least in man, psychotic episodes. The ability of some of the ataraxics to interfere with these actions has been in some measure an index of the drugs' phenotropic effect in the clinics (7, 8). Improvements and modifications of existing screening procedures and the introduction of newer methods of detecting and quantitating ataraxic activity are continually being reported (e. g., 9–11). Unfortunately, most of the laboratory methods for evaluating ataraxics have been far from satisfactory. Needless to say, a clear cut antihallucinogenic effect in laboratory animals that correlates well with a similar action in the psychotic patient would be of inestimable value in screening and assay procedures.

Apomorphine has never been widely acclaimed as having hallucinogenic properties. The drug is most familiar, of course, as an emetic and has been used for many years in the treatment of

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poisonings of various sorts and, in addition, has been employed in procedures designed to evaluate the antiemetic qualities of drugs (e. g., 12–14). However, in no other animal except the pigeon has there been reported a response to apomorphine that is so reminiscent of an active hallucination. The pecking syndrome is behaviorally distinct, readily quantitated, and reproducible. While it is perhaps presumptive to brand the pecking syndrome as hallucinatory, its characteristics suggest "higher center" activity that, with license, might be considered a hallucination. Whether or not the response is indeed hallucinatory and a function of "higher center" activity remains to be determined as does its relationship to mammalian responses induced by LSD and other psychomimetics.

It is suggested that the ability of ataraxic premedicants to block, inhibit, or potentiate the apomorphine response may throw additional light on the nature of the syndrome. The present communication describes the effect of selected central nervous system depressants and ataraxics on the apomorphine-induced pecking syndrome.

METHOD

Selection of Animals.—Adult pigeons (450–650 Gm.) of both sexes were employed in these experiments. Each was tested for apomorphine sensitivity by receiving an initial dose of the hydrochloride, 0.5 mg./Kg., i. p. Those few birds that were found to be refractory were discarded so that all pigeons finally selected as experimental subjects exhibited the distinct pecking syndrome in response to this dose of apomorphine.

Repeated Injection.—During the course of the investigation the same birds were sometimes used two, three, and infrequently four times. Since two to three weeks always elapsed between injections received by any one bird it was felt that there was little likelihood that the repeated use would modify the responses measured. Nevertheless, in order to ascertain whether or not tolerance, increased sensitivity, or Pavlovian conditioning does result from repeated administration of apomorphine and conceivably influence the data being collected, a group of six pigeons were injected with apomorphine hydrochloride, 0.5 mg./Kg., i. p., every third day (seventy-two hours) for a period of five weeks. The cumulative pecking response was measured periodically. On the fifteenth and thirty-fourth days the birds received saline injections in order to detect evidence of conditioning.

Controls.—Forty-nine birds having established apomorphine sensitivities served as controls. These pigeons received as the premedicating drug, 0.5 ml. of 0.9% sodium chloride solution prior to the apomorphine injection.

Drugs and Dosage.—The same dose of apomorphine hydrochloride was employed throughout the experiment: 0.5 mg./Kg. Ten drugs were selected as premedicants (Table I). Although all, except meprobamate and perphenazine, were administered in the form of a salt, dosage was calculated in terms of the base (or acid). Since comparative efficacy of these drugs was to be determined, the premedicants were administered in equimolar concentration: 2×10^{-4} moles per kilogram of body weight. All substances were administered intraperitoneally.

TABLE I.—EFFECT OF PREMEDICANTS ALONE ADMINISTERED INTRAPERITONEALLY

Drug	Dose Range, mg./Kg.	n	Response
Nalorphine hydrochloride	0.1–10.0	9	None
Morphine sulfate	0.1–10.0	12	Mild ataxia at 10.0
Pentobarbital sodium	1.0–10.0	6	None
Meprobamate	0.1–10.0	9	None
Hydroxyzine hydrochloride	0.1–10.0	9	Unusually quiet
Mepazine hydrochloride	0.1–10.0	9	Light sedation at 10.0
Promazine hydrochloride	0.1–10.0	12	2 vomited at 10.0
Chlorpromazine hydrochloride	1.0–20.0	18	Light sedation at 20.0
Perphenazine	0.1–10.0	9	None
Prochlorperazine ethanesulfonate	0.5–20.0	12	None

Premedication Alone.—In preliminary experiments the ten premedicants were administered in a variety of doses to groups of pigeons for the purpose of detecting gross effects such as sedation, excitation, and emesis.

Premedication Plus Apomorphine.—Fifteen minutes after injection of the test drugs, apomorphine was administered and the pigeons' responses monitored during the following two hours. Only rarely did the duration of a pecking episode exceed eighty minutes. It is felt that of the pecking syndrome characteristics quantitated, the cumulative (total) responses convey the most meaningful information and these therefore represent the measurement data recorded for subsequent analysis.

RESULTS

Interpolation from established dose-response data on the pecking syndrome (2) yielded expected or calculated values for pecking syndrome characteristics obtained from unpremedicated pigeons. Against these calculated values were compared the actual data obtained from two samples of birds one of which served as the control for this experiment (sample B). Data from the other group (sample A) were collected several months prior to this. Thus an opportunity is available to make observations on the reliability of these measurements. Actual and calculated values are presented in Table II. With the exception of the incidence (per cent of an arbitrary sampling of pigeons exhibiting the syndrome) all characteristics are in excellent agreement. The actual sample values for incidence

TABLE II.—RELIABILITY OF VARIOUS PECKING SYNDROME STATISTICS (APOMORPHINE HYDROCHLORIDE, 0.5 mg./Kg., i. p.)

	Calculated[a]	Sample A[b]	Sample B[c]
n	...	23	49
Incidence, %	98.5	82.1[d]	86.0[e]
Mean latent period ± S. E., min.	8.3	8.3 ± 1.1	8.7 ± 0.7
Mean duration ± S. E., min.	56	57 ± 3.0	55 ± 2.2
Mean maximum pecks per min. ± S. E.	108	110 ± 6.0	105 ± 15.1
Cumulative (total) pecks ± S. E.	3,300	3,354 ± 299	3,948 ± 121

[a] Interpolated from data presented in a previous communication (2). [b] August–September 1959. [c] January–February 1960. [d] In this instance, n = 28 (23/28 = 82.1%). [e] In this instance, n = 57 (49/57 = 86.0%).

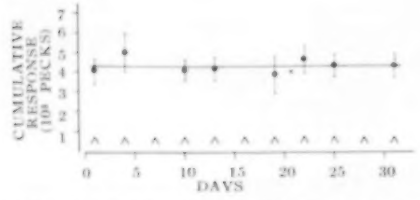

Fig. 1.—Effect of repeated injections of apomorphine hydrochloride on the cumulative pecking response. Arrows indicate frequency of drug administration. Each point represents the mean (±S. E.) of six pigeons.

were in both instances lower than the calculated value. Since nonreactors were always discarded as unsuitable for further experimentation, this disparity between calculated and actual incidence in no way affects this investigation. More important is the fact that there is such close agreement among all other measurements. No significant difference ($p > 0.05$) was found to exist between sample values.

Repeated injections of apomorphine were found to produce no evidence of either tolerance or increased sensitivity during a thirty-one day period (Fig. 1). The frequency of injection as well as the total number of injections were far in excess of those given to the experimental animals. Thus is eliminated the more serious objection to the use of the same animals more than once.

Further, it was observed that a conditioned response failed to develop until some time after a two-week period of injections had elapsed. Administration of saline on the fifteenth day elicited no response (i. e., no conditioning). However, following saline injection on the thirty-fourth day, three of the six pigeons exhibited weak pecking activity (287 ± 164).[1]

[1] Cumulative pecks, mean ± S. E.

The effects of the premedicants alone are described in Table I. Obvious effects were noted following the use of several of the drugs although only at the higher doses employed. The highest single dose devoid of all marked effects for all drugs was equivalent to about 2×10^{-4} moles (of base or acid) per Kg. This dose was chosen as the standard premedicant concentration.

In Table III is presented a summary of the effect of each premedicant on the cumulative pecking response accompanied by p values for the corresponding premedicated-control group pairs. A significant difference between pairs exists if $p < 0.05$. Of the drugs tested, hydroxyzine, chlorpromazine, perphenazine, and prochlorperazine exerted a highly significant diminution of pecking activity. Further analysis (Table IV) indicated that chlorpromazine, prochlorperazine, and perphenazine were equally effective (i. e., no significant difference among them) although all were significantly more effective than hydroxyzine.

Similarly, the incidence of the pecking syndrome was most severely reduced in the groups receiving these four premedicants. Most striking were the effects of the three chlorphenothiazines (Fig. 2, *A*).

The emetic activity of apomorphine in pigeons is ordinarily unimpressive. The intensity of vomiting produced by 0.5 mg./Kg. is considered trivial even though 12% of the control group exhibited some signs of emesis. When it occurs, the vomiting bout consists of one to three episodes each lasting ten to fifteen seconds. This is the extent of apomorphine-induced emesis. Several of the premedicants were observed to increase both the incidence (Fig. 2, *B*) and the severity of vomiting due to apomorphine. Nalorphine, meprobamate, and mepazine were most outstanding in this respect. Hydroxyzine increased the incidence but not the severity.

TABLE III.—EFFECT OF PREMEDICATION ON THE APOMORPHINE-INDUCED PECKING SYNDROME

Drug	Dose,[a] mg./Kg.	Cumulative (Total) Response[b]	n	p
Saline (control)	...	3,948 ± 121	49	...
Nalorphine	6.23	3,707 ± 650	12	>0.8
Morphine	5.71	3,169 ± 684	12	>0.3
Pentobarbital	4.51	4,230 ± 686	12	>0.1
Meprobamate	4.36	5,336 ± 393	12	>0.05
Hydroxyzine	7.48	1,053 ± 337	12	<0.001
Mepazine	6.21	5,000 ± 984	12	>0.2
Promazine	5.69	3,004 ± 243	12	>0.2
Chlorpromazine	6.37	102 ± 67	12	<0.001
Perphenazine	8.08	0	11	<0.001
Prochlorperazine	7.48	33 ± 30	12	<0.001

[a] 2×10^{-4} moles/Kg. [b] Mean ± S. E.

TABLE IV.—p VALUES FOR PAIRS OF THOSE DRUGS [illegible] CTING AGAINST THE APOMORPHINE RESPONSE

	Hydroxyzine	Perphenazine	Prochlorperazine
Chlorpromazine	<0.05	>0.1	>0.3
Prochlorperazine	<0.02	>0.3	
Perphenazine	<0.02		

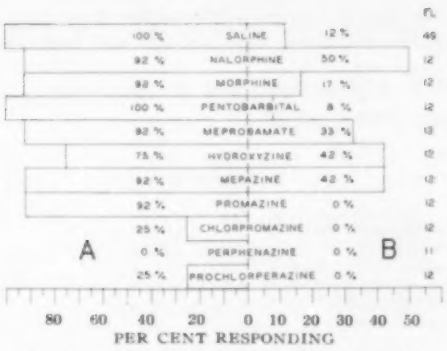

Fig. 2.—Incidence of the apomorphine-induced pecking syndrome (*A*), and emesis (*B*), in groups of pigeons premedicated with 2×10^{-5} moles per Kg. of the listed drugs.

It should be recalled that these drugs, when administered alone, are not in themselves emetic. No vomiting was observed to occur in those birds receiving chlorpromazine, promazine, prochlorperazine, and perphenazine as premedicants.

DISCUSSION

The first point that deserves comment is the observation that under the circumstances of the present experiment neither tolerance nor increased sensitivity to apomorphine was found to develop in pigeons. Tolerance to the excitatory action of the drug in mammals has been the subject of conflicting reports in the past. With respect to the emetic component of apomorphine's activity, investigators failed to find any evidence of tolerance in man (15) or the dog (16–18); found that the dog became tolerant (19, 20); reported that increased susceptibility appears to develop with repeated administration (21). Thus every possibility seems to boast advocates. Certainly it is recognized that, if given early enough and in large enough quantity, a second dose of apomorphine will serve to depress the vomiting center and thereby render ineffective any concomitant CTZ stimulation that may occur. Under such a circumstance, an apparent tolerance to apomorphine will be manifest. It would appear likely, then, that different conditions surrounding drug administration (dose, route, frequency of injection, etc.) may explain the variability of results reported. In any case, with repeated injections administered under conditions previously described, the pecking syndrome characteristics remained unaltered within the limits of significance.

Neither morphine nor its derivative nalorphine were found to exert any influence on the pecking syndrome in the doses employed. Nalorphine, however, did increase the incidence of emesis due to apomorphine. An emetic potentiating action of nalorphine has not been observed in mammals. Of particular interest is the fact that nalorphine has been reported to have no effect on apomorphine-induced emesis in the dog although it has been observed to abolish the emesis produced by morphine (22). This appears curious inasmuch as both apomorphine and morphine are believed to exert their emetic actions by the same general mechanism (23). The potentiation of apomorphine emesis by nalorphine in pigeons serves to add to the confusion, although it must be recognized that the neurological mechanisms involved here may be quite different in the bird from those in the mammal.

The antagonism of apomorphine-induced emesis by morphine can be readily demonstrated in several animals and is believed to be the result of central depression (24). However, in a dose that would be expected to exert just such an inhibitory effect in dogs, morphine fails to influence the pecking syndrome in pigeons. Further, morphine does not share with apomorphine the ability to initiate the syndrome nor does it produce the marked narcosis seen in so many other animals when administered in doses of 5–10 mg./Kg. It would appear that pigeons are considerably more resistant to both the stimulant and depressant actions of morphine than are the more familiar laboratory animals.

Pentobarbital was similarly ineffective in interfering with the pecking response. In sufficient quantities this drug, too, will block apomorphine emesis in mammals but only in amounts that will produce distinct hypnosis (25). In such concentrations, pentobarbital can be expected to exert a cerebral depressant effect. The hypothesis that the pecking syndrome is a function of "higher center" activity might be accompanied by the suspicion that a CNS depressant such as pentobarbital may prove to be more effective as a pecking syndrome antagonist than an antiemetic. However, such appears not to be the case.

Meprobamate, widely employed as a muscle relaxant and in the treatment of mild psychoses, had no demonstrable effect on the syndrome. It is the experience of investigators that meprobamate is considerably less effective than the chlorphenothiazines in treating psychiatric disorders (26) and, as a result, has been classified by Seneca (27) as a "mild" ataraxic. Although similarly classed, hydroxyzine did significantly depress pecking activity. In addition, both drugs were found to increase the incidence of emesis in birds for which effect there is, at present, no explanation. Although meprobamate apparently exerts no important influence on drug-induced emesis in mammals, hydroxyzine is reported as possessing distinct antiemetic action in these animals (27, 28). It has been observed on other occasions that antiemetic action in mammals is not paralleled by similar action in pigeons (1).

The ability of the five phenothiazines to inhibit the apomorphine-induced pecking syndrome appears to parallel, to some extent, the drugs' antiemetic and ataraxic actions in mammals when employed in comparable doses. The substitution of chlorine on the phenothiazine nucleus apparently confers increased apomorphine antagonism with respect to both emesis in dogs (29) and pecking in pigeons. Hydroxyzine, the only other substance of the group studied that exhibited apomorphine inhibition is, interestingly enough, also chlorinated. The efficacy of hydroxyzine as a pecking syndrome antagonist is, however, inferior to chlorpromazine, prochlorperazine, and perphenazine, three substances whose activities as both ataraxics and anti-

emetics are well established (e. g., 30–32). These chlorinated phenothiazines, then, proved to be the most active apomorphine antagonists in pigeons.

Mepazine and promazine, the two nonchlorinated phenothiazines devoid of antiapomorphine activity in pigeons have been shown to have no antiemetic action in dogs (28) although these substances are employed as both ataraxics and (to a lesser extent) antiemetics, and presumably are effective as such in man.

The question may reasonably arise as to whether pecking inhibition is a function of the drugs' hallucinolytic property or more specifically an antiapomorphine effect. Although the problem is an important one from the point of view of mechanism and invites conjecture, until we are able to induce a similar "hullucination" in pigeons by other means (and determine the effectiveness of our ataraxics against it) it is not possible at this time to answer the question with certainty.

SUMMARY

1. No significant alteration of pecking syndrome characteristics results from repeated injections of apomorphine over a five-week period.

2. From fifteen to thirty-four days of repeated apomorphine injections are required to develop a conditioned pecking response.

3. Of the premedicants employed (in doses of 2×10^{-5} moles/Kg.), only hydroxyzine, chlorpromazine, prochlorperazine, and perphenazine significantly inhibited the syndrome.

4. The three chlorinated phenothiazines were significantly more effective than hydroxyzine.

5. Nalorphine, morphine, pentobarbital, meprobamate, and the nonchlorinated phenothiazines, mepazine and promazine, failed to influence the syndrome.

6. Apomorphine emesis in pigeons which is ordinarily mild in intensity, brief in duration, and low in incidence, is potentiated by nalorphine, mepazine, meprobamate, and hydroxyzine.

7. Significance of some of these responses is discussed.

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Studies on Local Anesthetics XXVI

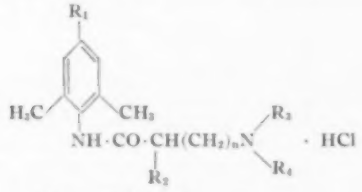
Influence of the Lateral Side Chain on the Anesthetic Activity of Substituted Basic Acetanilides

By RUDOLF DOFEK, ALEŠ SEKERA†, and ČENĚK VRBA‡

A series of twenty-two substituted basic acetanilides, in which the chemical structure of the acyl substituents and amino groups were modified, has been prepared. At the same time the synthesis of ω-diethylaminothioaceto-2,6-xylidide and -2,4,6-mesidide has been reported. The anesthetic activity of these compounds as determined by surface and infiltration methods has been reported and the results of toxicity tests have been summarized.

THE METHYLATED analog of lidocaine,[1] ω-diethylaminoaceto-2,4,6-mesidide, already described by Löfgren in 1946 (1), has recently awakened the interest of other research groups (2–7). The advantageous results of the pharmacological tests and clinical trials of this substance as well as, under certain circumstances, its better accessibility have led to its introduction into practical medicine under the name of Mesokain (8).

These facts indicated the desirability of preparing a series of basic acetanilides of general formula I, in which the influence of the structural



R_1 = H, CH_3
R_2 = H, CH_3–C_3H_7
R_3, R_4 = H, alkyl, alkylene, etc.
n = 0, 1

changes of the lateral side chain on the local anesthetic activity might be investigated. These studies included the influence on activity effected by ramifying substitutions around the acyl radical and chemical modifications of the amino group. Simultaneously, the thioanalogs of lidocaine and Mesokain[2] were prepared. The preparation and pharmacological evaluation of the substances described in this study are the continuation of previous papers from this laboratory on substituted basic acetanilides (7, 11) and thioderivatives of the series of substituted ω-aminoacetophenones (12).

The compounds were prepared according to the method previously used (7, 11), i.e., by the reaction of the corresponding halo-acetanilides with diethylamine. The thioacetanilides were obtained by action of phosphorus pentasulfide on corresponding acetanilides.

EXPERIMENTAL[3]

Diethylaminoacetomesidides (I; S 352–S 395).—These were prepared in the majority of cases according to the method previously used (7, 11) by the reaction of the corresponding chloroacetanilides with the diethylamine in boiling anhydrous benzene.

In the cases in which the reactivity of the starting products was reduced by the steric factor resulting from α-alkylation, the reaction was carried out in autoclave at 140–160° (S 383, S 388, S 390–S 392). For a similar reason the more reactive bromo derivatives were used as the starting halo-acetanilide intermediate.

The β-diethylaminopropiono-2,4,6-mesidide (S 395) was prepared at laboratory temperature (forty-eight days) in order to avoid the formation of an acrylomesidide by-product.

The ω-(4-carbethoxypiperazino-1)-acetomesidide (S 385) was prepared by the reaction of equivalents of chloroacetomesidide and 1-carbethoxypiperazine in presence of sodium bicarbonate in boiling ethanol.

The starting halo-aceto-2,4,6-mesidides were prepared by the reaction of chlorides of corresponding halo-aliphatic acids with mesidine in acetic acid over sodium acetate (1).

Thioacetanilides (S 322 and S 323).—These were prepared by the reaction of the corresponding acetanilides with the phosphorus pentasulfide in anhydrous pyridine (13).

The final substances were converted to the hydrochlorides and isolated as such. These were precipitated from anhydrous ether or benzene solution of

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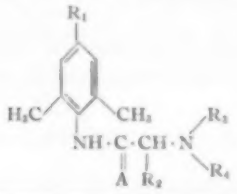
[1] The trade name of lidocaine is Xylocaine (Astra).

[2] After having finished the experimental part of this study we discovered the existence of the studies of Löfgren's group (9, 10) who simultaneously with us prepared five derivatives (S 322, S 323, S 352, S 357, and S 374).

[3] All melting points were determined on the Kofler block and are corrected. Microanalyses were carried out by Mrs. Kleinová-Parolková.

TABLE I.—SUBSTITUTED BASIC ACETANILIDE AND THIOACETANILIDE HYDROCHLORIDES



No.	R_1	A	R_2	R_3	R_4	M. p., °C.	Solubility, H_2O, %	N, % Calcd.	N, % Found	Cl, % Calcd.	Cl, % Found
S 322	H	S	H	C_2H_5	C_2H_5	162[a]	...	9.77	9.88	...	...
S 323	CH_3	S	H	C_2H_5	C_2H_5	160[b]	...	9.31	9.38	...	...
S 352	CH_3	O	H	C_4H_9	H	247[c]	1.7	9.84	9.75	...	...
S 354	CH_3	O	H	C_6H_{13}	H	199	0.45	8.95	8.86	11.33	11.30
S 357	CH_3	O	H	C_8H_{11}	H	254[d]	2	9.01	8.98	...	...
S 359	CH_3	O	H	$C_6H_5CH_2$	H	205	0.9	8.79	8.72	11.12	10.88
S 371	CH_3	O	H	C_4H_9	C_4H_9	126	3.4	8.22	8.25	10.40	10.38
S 381	CH_3	O	H	C_6H_5	C_2H_5	163	0.09	8.42	8.23	10.65	10.56
S 384	CH_3	O	H	$CH_2CH_2CH_2CH_2$		213–214	(40–50)	9.91	9.90	12.54	12.57
S 374	CH_3	O	H	$CH_2CH_2CH_2CH_2CH_2$		196[e]	(38)	9.44	9.35	...	...
S 375	CH_3	O	H	$CH(CH_3)\cdot CH_2CH_2CH_2CH_2$		188	(40–50)	9.01	9.01	11.41	11.47
S 376	CH_3	O	H	$CH_2CH(CH_3)\cdot CH_2CH_2CH_2$		104	(5)	9.01	9.00	11.41	11.42
S 377	CH_3	O	H	$CH_2CH_2CH(CH_3)\cdot CH_2CH_2$		217–219	(>50)	9.01	8.82	11.41	11.32
S 383	CH_3	O	H	$CH(CH_3)\cdot CH_2CH_2CH_2CH(CH_3)$		261	4.7	8.60	8.61	10.89	10.78
S 388	CH_3	O	H	$CH(CH_3)\cdot CH_2CH(CH_3)\cdot CH_2CH(CH_3)$		175	4.3	8.27	8.15	10.46	10.45
S 378	CH_3	O	H	$CH_2CH_2O\cdot CH_2CH_2$		250	(12)	9.38	9.29	11.87	11.74
S 379	CH_3	O	H	$CH_2CH_2S\cdot CH_2CH_2$		225	6.4	8.90	8.67	10.90	11.17
S 385	CH_3	O	H	$CH_2CH_2N(COO\cdot C_2H_5)\cdot CH_2CH_2$		132	6	11.39	11.30	9.61	9.45
S 390	CH_3	O	CH_3	C_2H_5	C_2H_5	212	(>50)	9.37	9.29	11.86	11.90
S 391	CH_3	O	C_2H_5	C_2H_5	C_2H_5	216–217	16	8.95	8.89	11.34	11.47
S 392	CH_3	O	C_3H_7	C_2H_5	C_2H_5	175	(2–5)	8.57	8.54	10.85	10.90
S 395	CH_3		$CO\cdot CH_2CH_2N(C_2H_5)_2$			163	(>50)	9.37	9.28	11.86	11.05

[a] Lüning (9) m.p. 106.5–107°. [b] Lüning (9) m. p. 121°. [c] Literature (10) m. p. 237–238°. [d] Literature (10) m. p. 261–262°. [e] Literature (10) m. p. 197–199°. [f] Compound mentioned in patent literature (16) but constants are not given.

bases by addition of ethereal hydrogen chloride. The yields were generally good (in preparation of the bases, 50–90%).

The melting points and analyses of the substances prepared are included in Table I.

The solubilities of the hydrochlorides were determined by the mercurimetric titration of chloride ion in solutions saturated at room temperature. The results are given in Table I. The values in parentheses are the results of preliminary experiments in test tubes.

PHARMACOLOGY

The relative activity of the compounds in surface anesthesia (rabbit cornea, 0.01*M* cocaine as standard) and infiltration anesthesia (intradermal application to guinea pigs, 0.02*M* procaine as standard) was calculated from the molar concentration experimentally found to give the same effect as the standard. The method has been described in detail by Vrba and Sekera (14).

The toxicity was studied according to Kärber (15) by determining the LD_{50} in white mice (strain H) by subcutaneous application.

The results are given in Table II.

DISCUSSION AND SUMMARY

Twenty-two derivatives of the lidocaine series were prepared and tested for local anesthetic activities and toxicity.

The following correlations between the anesthetic activity, acute toxicity, and chemical structure of the compounds studied can be made:

1. Ramifications of the acyl chain in general appear to accentuate the anesthetic action in surface anesthesia as in infiltration anesthesia; this effect increases with the prolongation of the lateral side chain

TABLE II.—PHARMACOLOGICAL PROPERTIES

Substance	Relative Activity — Surface Anesthesia	Relative Activity — Infiltration Anesthesia	LD_{50} s. c. mg./Kg.
S 322	0.17	0.77	820
S 323	0.05	0.66	>14,000
S 352	0.62	2.6	270
S 354	4.9	1.2	280
S 357	2.7	8.3	170
S 359	2.2	4.1	630
S 371	9.3	5.1	>4,500
S 381	Sparingly soluble[a]	Sparingly soluble[a]	Sparingly soluble[b]
S 384	0.45	2.1	540
S 374	0.43	1.5	320
S 375	0.6	4	240
S 376	2.6	8.4	580
S 377	2.8	5.5	570
S 383	3.3	3.4	480
S 388	3	4.4	940
S 378	(0.2–0.3)	3.1	660
S 379	Sparingly soluble[c]	Sparingly soluble[c]	>2,300
S 385	0.07	1.1	710
S 390	0.76	3.6	370
S 391	1.2	4	250
S 392	9.3	7.7	135
S 395	0.59	1.2	390
S 202 (lidocaine)	0.24	1.4	370
S 203 (Mesokain)	1	2.5	350
Cocaine	1	3.6	125
Procaine	0.15	1	630

[a] No action after application of 0.00303 *M* solution. [b] $LD_{50} \geq 90$ (saturated solution). [c] No action after application of 0.2 *M* solution.

(S 390–S 392). The prolongation of the nonramified chain, if it is possible to judge from a single example (S 395), does not seem to offer any advantage.

2. The N-monosubstituted derivatives (S 352, S 354, S 357, and S 359) were seen to be generally more active than the model substance, Mesokain (S 203). It must be noted, however, that they are also more toxic, with the exception of the benzyl derivative (S 359) which seems to be the most advantageous of this subgroup.

3. The only aliphatic substituted derivative, dibutylaminoacetomesidide (S 371), is almost ten times more active in surface anesthesia and two times more active in infiltration anesthesia than Mesokain, and more than ten times less toxic than this model substance.

4. Replacing of the diethylamino group in the Mesokain molecule by a cycloaliphatic amine was seen to be advantageous and therefore received greater attention.

Replacing by morpholine (S 378), carbethoxypiperazine (S 385), and especially by thiomorpholine (S 379) produces, indeed, a lowering of toxicity but in general also a parallel fall in activity.

Replacing by pyrrolidine (S 384) lowers toxicity and activity. Replacing by nonsubstituted piperidine (S 374) is also not too advantageous; however, in the series of methylpiperidine derivatives fall several substances having the best activity-toxicity ratio of the entire series of compounds described in this paper.

Among the monomethylpiperidino derivatives the most advantageous is the derivative of β-picoline (S 376), which is more than two times more active in surface anesthesia and three times more active in infiltration anesthesia than Mesokain, and at the same time nearly two times less toxic. The derivatives of 2,6-lupetidine (S 383) and 2,4,6-copellidine (S 388) were found to be even more interesting, being approximately three times more active in surface anesthesia and also more active in infiltration anesthesia than Mesokain, and simultaneously less toxic. The favorable activity-toxicity ratio of all these substances stand out also in comparison with cocaine and procaine.

5. Substituting sulfur for oxygen in the series studied was seen to be much less favorable than in theFalicaine series (12). Thio-lidocaine (S 322) as well as thio-Mesokain (S 323) were, indeed, found to be less toxic than the corresponding oxygenated substances, but also were less active. This effect is even more pronounced in the case of substitution of sulfur for the oxygen of morpholine in the lateral side chain of the substance S 378: the thiomorpholino derivative (S 379) is only very slightly toxic but practically without anesthetic action.

A more thorough study of the most promising substances is under way. It will be published elsewhere along with the results of the study of the relations between the anesthetic activities of the substances described in this paper with their physicochemical properties such as surface activity, liposolubility, ability to coagulate colloids, basicity, etc.

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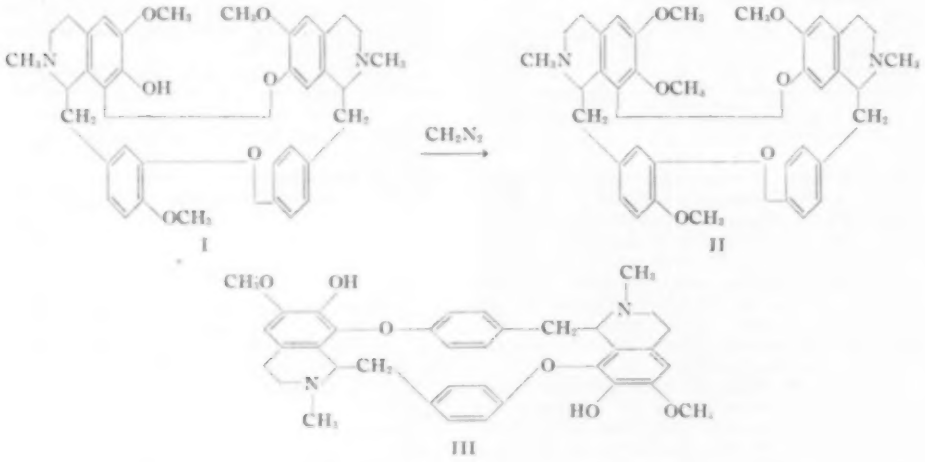
Menispermaceae Alkaloids II

The Alkaloids of *Cyclea peltata* Diels

By S. MORRIS KUPCHAN, NAOKATA YOKOYAMA, and B. S. THYAGARAJAN

The roots of *Cyclea peltata* Diels from Madras yielded *d*-tetrandrine, *dl*-tetrandrine, *d*-isochondrodendrine, and fangchinoline. Preliminary pharmacological study of the petroleum ether-extractable alkaloids, of the methanol-extractable alkaloids, and of the methiodides prepared from the latter mixtures showed that all produce activity similar to that seen with *d*-tubocurarine.

CYCLEA PELTATA Diels is a menispermaceous plant used in India against "colicky pains in the stomach, fevers, vomiting, skin conditions, diarrhea, cardiac pains, burning feeling, pruritus, poisons, breathing difficulties, worms, colicks, internal tumors (?), 'gulma' colicks, in poisons taken internally ('gara'), and ulcers" (1). The drug has also been used in setting fractures and as an internal remedy in inflamed piles (1).



An earlier examination revealed the presence in *C. peltata* Diels of alkaloids (0.51 per cent), fixed oils (8.0 per cent), quercitol, and traces of a coloring matter (1). The present report describes a preliminary study of the alkaloids extracted from air-dried roots of *C. peltata* Diels from Madras.

Coarsely ground plant was extracted successively with petroleum ether, methanol, 1.5 per cent triethylamine in methanol, and 1.5 per cent hydrochloric acid solution. Each extract was processed for alkaloid content by the procedure summarized in Fig. 1, whereby a 2.0 per cent total yield of crude alkaloids was obtained. The crude fractions obtained were either crystallized directly or subjected to chromatography, whereby separation into crystallizable fractions was effected. The isolation procedure afforded *d*-tetrandrine as the principal isolable alkaloidal constituent (0.4 per cent) and *d*-isochondrodendrine (0.045 per cent), fangchinoline (0.025 per cent), and *dl*-tetrandrine (0.008 per cent) as lesser isolable constituents. Preliminary pharmacological study of the petroleum ether-extractable alkaloids (fraction A), of the methanol-extractable alkaloids (fraction B), and of the methiodides prepared from the latter mixtures showed that all produce activity similar to that seen with *d*-tubocurarine.[1]

d-Tetrandrine (II) and *d*-isochondrodendrine (III) were characterized by direct comparison

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[1] We thank Mr. Edward Macko of the Smith Kline and French Laboratories, Philadelphia, Pa., for the pharmacological results reported herein.

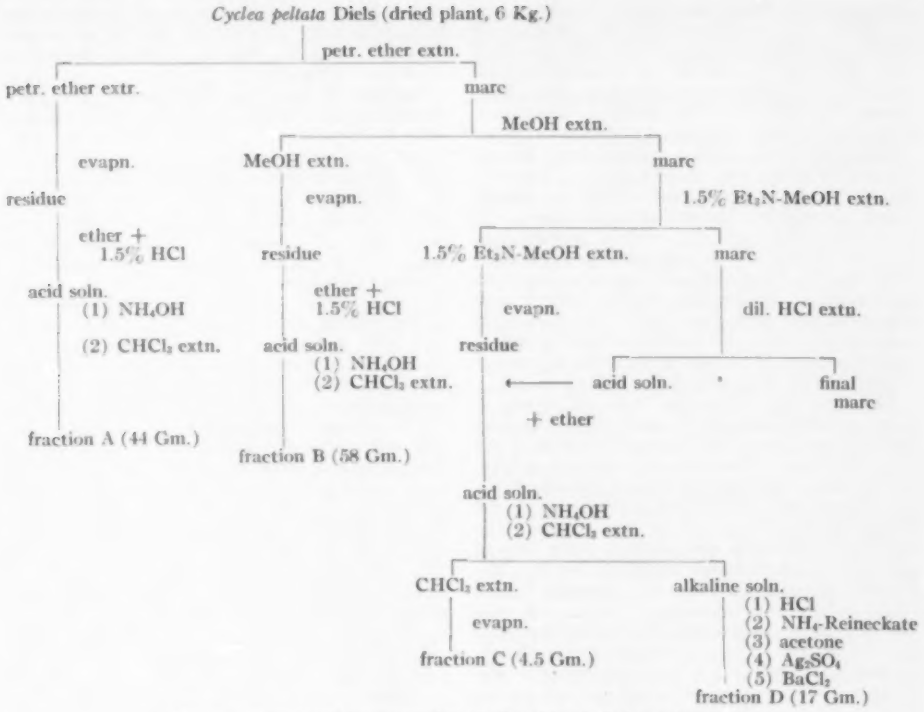

Fig. 1.—Flow sheet for separation of alkaloids of *Cyclea peltata* Diels.

with authentic samples of the alkaloids. Fangchinoline (I) was initially characterized by its physical constants and by its positive phenolic test with methanolic ferric chloride in spite of its insolubility in aqueous alkali. Confirmation for the identity of the compound was obtained by methylation with diazomethane to yield *d*-tetrandrine. *dl*-Tetrandrine was initially characterized by the identity of its infrared spectrum in chloroform with that of *d*-tetrandrine which accompanied optical inactivity and a higher melting point. Confirmation for the identity of the material was achieved by resolution via the respective *d*-10-camphorsulfonic acid salts into *d*-tetrandrine and its optical antipode, pheanthine.

d-Tetrandrine, fangchinoline, and *d*-isochondrodendrine have been isolated previously from drugs derived from menispermaceous plants (2). *d*-Tetrandrine was isolated from han-fang-chi and mu-fang-chi, obtained from plants from the Mukden area and appearing on the Chinese market (3–6) and also from *Stephania tetrandra* S. Moore of Formosa and *Menispermum dauricum* (7). Fangchinoline has also been isolated from han-fang-chi (4–6). *d*-Isochondrodendrine was isolated from the drug *Radix pareirae bravae* of the English market (8), from *Chondodendron platyphyllum* Miers, *Ch. microphyllum* (Eichl.) Moldenke (8), and *Ch. tomentosum* Ruiz and Pav. (9), and from *Cissampelos pareira* Linn. (10).

dl-Tetrandrine does not appear to have been isolated previously from natural sources. Indeed, *l*-tetrandrine (pheanthine) has apparently been isolated only from *Pheanthus ebracteolatus* (Presl.) Merrill of the family Anonaceae (11, 12) and the present work therefore constitutes the first isolation of *l*-tetrandrine from a plant of the family Menispermaceae.

EXPERIMENTAL

Melting points have been corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Infrared spectra were determined on a Baird double beam infrared recording spectrophotometer. Ultraviolet absorption spectra were determined in 95% ethanol on a Cary recording spectrophotometer (model 11 MS). Paper chromatography was conducted by the descending technique on Whatman No. 1 paper.

Extraction of Alkaloids from Cyclea peltata.

Separation into Main Fractions.—Coarsely ground *C. peltata*[2] (air-dried roots, 6 Kg.) from Madras was extracted continuously for a week with petroleum ether (Skelly B, b. p. 60–68°) in a Soxhlet-type extractor. The extraction was continued with a fresh charge of solvent. When the extraction was stopped, after a second week, the extract returning to the pot yielded upon evaporation a residue which did not give a positive test with Mayer's reagent. Evaporation of the whole extract under reduced pressure left a semisolid residue. The residue was triturated with 1.5% hydrochloric acid (2L.) and the insoluble portion was rejected. The acid solution was washed with ether (3L.) and made alkaline with ammonium hydroxide, and the precipitated base was extracted repeatedly with chloroform (4L.). The chloroform solution was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to yield 44 Gm. of fraction A (see Fig. 1).

The dried marc remaining from the petroleum ether extraction was next extracted continuously with methanol, with a fresh charge of solvent at the end of five days. When the extraction was stopped, after a total of ten days, the extract returning to the pot yielded upon evaporation a residue which did not give a positive test with Mayer's reagent. The methanol extract was concentrated under reduced pressure to a dark brown semisolid residue. The residue was triturated with 1.5% hydrochloric acid (3 L.) and the acid extractions were repeated until the extract no longer gave a positive test with Mayer's reagent. The acid extracts were combined, washed with ether (3 L.), made alkaline with ammonium hydroxide, and extracted with chloroform (5 L.). The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to yield a semisolid alkaloidal residue (fraction B, 58 Gm.).

The marc remaining from the methanol extraction was next extracted continuously with 1.5% triethylamine in methanol for twelve days. When the extraction was stopped, the extract returning to the pot yielded upon evaporation a residue which did not give a positive test with Mayer's reagent. The marc remaining from this extraction was next extracted with 1.5% hydrochloric acid (5 L.) by percolation. The triethylamine-methanol extract was evaporated to dryness, and the residue was triturated with the hydrochloric acid extract of the final marc. After rejecting acid-insoluble material, the acid solution was washed with ether, made alkaline with ammonium hydroxide, and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to yield 4.5 Gm. of alkaloid fraction C. The alkaline solution was reacidified with hydrochloric acid and treated with a saturated Reinecke salt solution. The precipitated salt was dissolved in acetone and the solution was clarified by filtration and then treated with saturated aqueous silver sulfate solution to complete precipitation. The silver Reineckate was removed by filtration, and the filtrate was treated with barium chloride solution to complete precipitation. After removal of the barium sulfate by centrifugation, the supernatant solution was evaporated to dryness under reduced pressure to yield 17 Gm. of quaternary chloride fraction D. Studies of this fraction will be reported at a later date.

Isolation of Alkaloids.—*Fraction A.*—The crude alkaloid fraction (44 Gm.) was extracted with boiling ether for three hours. The ether extract was evaporated to dryness and the residue was crystallized from acetone to yield prisms (12.0 Gm.). Repeated recrystallization from ether (during which the ether-insoluble solid was separated) afforded prisms, m. p. 216–217°; $[\alpha]_D^{25}$ + 274° (c 2.16, $CHCl_3$); $\lambda_{max.}$ 282.5 mμ (ε 8,050); $\lambda_{min.}$ 258 mμ (ε 3,010).

Anal.—Calcd. for $C_{38}H_{42}N_2O_6$: C, 73.29; H, 6.80; N, 4.50. Found: C, 73.61; H, 6.70; N, 4.30.

The melting point was not depressed on admixture with an authentic sample of *d*-tetrandrine.[3] The paper chromatographic behavior (10) and infrared spectrum in chloroform solution were identical with those of the authentic *d*-tetrandrine sample.

The acetone mother liquor was evaporated to dryness and the residue was dissolved in chloroform and chromatographed on Florisil (600 Gm.). The column was developed by successive elution with chloroform, 1% acetone-chloroform, 10% acetone-chloroform, 30% acetone-chloroform, 60% acetone-chloroform, acetone, ethanol, and methanol. The 10% and 30% acetone-chloroform eluates gave mixtures of *d*-tetrandrine and a second alkaloid. Fractional crystallization from acetone ultimately gave 2.0 Gm. of *d*-tetrandrine and 0.50 Gm. of the second alkaloid, m. p. 237–238°; $[\alpha]_D^{25}$ + 258° (c 1.05, $CHCl_3$); $\lambda_{max.}$ 283 mμ (ε 8,450); $\lambda_{min.}$ 259 mμ (ε 3,160); picrate crystallized from alcohol, m. p. 186°; picrate crystallized from acetone, m. p. 225°. The alkaloid gave a positive test for phenols with methanolic ferric chloride solution and showed a medium intensity absorption band at 2.92 μ (OH). The aforementioned properties supported the identification of the alkaloid as fangchinoline (6). Confirmation for the identity of the alkaloid was obtained by methylation of fangchinoline in methanol-chloroform solution with diazomethane, whereupon *d*-tetrandrine was obtained (*cf.* 6).

The ether-insoluble portion of fraction A afforded 2 Gm. of *d*-tetrandrine and 100 mg. of fangchinoline by chromatography on Florisil followed by fractional crystallization as described above.

The ether-insoluble alkaloid accumulated during the repeated recrystallization of crude *d*-tetrandrine from ether was recrystallized from absolute ethanol to give colorless needles, m. p. 252–253°; $[\alpha]_D^{25}$ ± 0° (c 4.25, $CHCl_3$). The infrared and ultraviolet spectra and the paper chromatographic behavior of this material were identical to those of *d*-tetrandrine. These facts supported identification of the material as *dl*-tetrandrine, and led to the attempt to confirm the identity by resolution.[4]

Resolution of dl-Tetrandrine.—*d*-Tetrandrine *d*-10-camphorsulfonate was first prepared by dissolving in boiling absolute ethanol (20 cc.) *d*-tetrandrine (182 mg.) and *d*-10-camphorsulfonic acid (69 mg.).

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[2] We thank Rajaranga and Co., Madras, India, for gathering and forwarding the dried plant materials to us, and Dr. C. B. Sulochana, University Botany Laboratory, Madras, for confirming the identity of the plant.

[3] We thank Professor Masao Tomita, Kyoto University, Japan, for authentic samples of *d*-tetrandrine and *d*-isochondrodendrine.

[4] It is noteworthy that Kondo and Keimatsu (12) prepared a synthetic mixture of *d*-tetrandrine and *l*-tetrandrine and reported that the needles from methanol showed m. p. 252°; $[\alpha]^D = \pm 0°$ (c 4.82, $CHCl_3$).

Concentration and cooling yielded colorless needles (165 mg.) of *d*-tetrandrine *d*-10-camphorsulfonate, m. p. 237–238° (decompn.), $[\alpha]_D^{25}$ + 192° (c 0.88, methanol); $\lambda_{max.}$ 282 mμ; $\lambda_{min.}$ 258 mμ.

Anal.—Calcd. for $C_{48}H_{58}N_2O_{10}S \cdot {}^1/_2 H_2O$: C, 66.71; H, 6.88; N, 3.23. Found: C, 66.70; H, 7.04; N, 3.46.

The resolution was accomplished by treating a solution of *dl*-tetrandrine (182 mg., m. p. 252–253°) in chloroform (2 cc.) and absolute ethanol (30 cc.) with *d*-10-camphorsulfonic acid (69 mg.). The solution was concentrated to about 3 cc., seeded with *d*-tetrandrine *d*-10-camphorsulfonate, and cooled, whereupon colorless needles (63 mg.), m. p. 237–238° (decompn.), separated. The camphorsulfonate salt (25 mg.) was dissolved in 1% hydrochloric acid (4 cc.) and the solution was washed with chloroform and then made alkaline with ammonium hydroxide. The liberated base was extracted with chloroform and the chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was crystallized from absolute ethanol to yield 15 mg. of prisms shown to be *d*-tetrandrine by m. p., mixed m. p., infrared spectrum, and optical rotation in chloroform solution. The mother liquor of the *d*-tetrandrine *d*-10-camphorsulfonate yielded two further crops of crystals (70 mg.) on cooling; these materials were shown to be mixtures by m. p. and optical rotation. The filtrate was finally evaporated to dryness and the residue was treated to liberate free base as described above. The liberated free base was crystallized from ether (28 mg.), and repeated recrystallization from ether afforded colorless needles (15 mg.), m. p. 215–217°; $[\alpha]_D^{27}$ − 247° (c 0.71 $CHCl_3$); $\lambda_{max.}$ 282 mμ (ε 8,250); $\lambda_{min.}$ 255 mμ (ε 3,060). The infrared spectrum in chloroform and the paper chromatographic behavior of this material were identical with those of *d*-tetrandrine. These facts and the negative rotation of the alkaloid strongly supported identification of the alkaloid as *l*-tetrandrine (11, 12).

Fraction B.—The crude alkaloid fraction (58 Gm.) was divided into ether-soluble (29 Gm.) and ether-insoluble fractions as above. The ether-extractable alkaloid fraction yielded 10.2 Gm. of *d*-tetrandrine and 0.90 Gm. of fangchinoline by chromatography on Florisil followed by fractional crystallization, as described above. The ether-insoluble fraction in chloroform was likewise chromatographed on Florisil. The methanol eluate yielded a crystalline residue (900 mg.) which was recrystallized from pyridine-methanol to yield microcrystals (600 mg.), m. p. 314–315° (decompn.), $[\alpha]_D^{25}$ + 55° (c 0.94 pyr.); $\lambda_{max.}^{0.1\ N\ HCl}$ 273 mμ (ε 4,500), 282 mμ (ε 4,400); $\lambda_{min.}^{0.1\ N\ HCl}$ 257 mμ (ε 3,050); 277 mμ (ε 4,200). The melting point was not depressed on admixture with an authentic sample of *d*-isochondrodendrine.[3] The paper chromatographic behavior and infrared spectrum (Nujol mull) were identical with those of the authentic *d*-isochondrodendrine sample.

Fraction C.—Crystallization of the crude alkaloid fraction from methanol gave 2.1 Gm. of *d*-isochondrodendrine. The methanolic mother liquor showed paper chromatographic behavior which suggested the presence of another alkaloid, but attempts to crystallize the latter substance were unsuccessful.

PHARMACOLOGICAL RESULTS

Preliminary pharmacological tests were performed on the petroleum ether-extractable alkaloids (fraction A), the methanol-extractable alkaloids (fraction B), and on the methiodides prepared from each of the latter. Orally in mice, fraction A produced depression of motor activity, ptosis, mydriasis, and somnolence following doses of 500 or 1,000 mg./Kg. No significant alterations in body temperature or pain threshold were observed. These alkaloids appear to possess weak but nonspecific sedative properties at relatively high doses. Intraperitoneally, fraction B caused decreased activity and hind leg weakness at 50 mg./Kg. Higher doses caused depression, hind leg weakness, somnolence, low posture, and delayed death at 200 mg./Kg. Orally, very weak gross effects occurred even at doses of 2,000 mg./Kg. The methiodide mixture derived from fraction B produced effects characteristically seen with *d*-tubocurarine. Intraperitoneally, 5 mg./Kg. caused ataxia, absence of grasp and pinnal reflexes, marked hypotonia, gasping, clonic and asphyxial convulsions, and death. As seen with *d*-tubocurarine, a very narrow range exists between doses causing minimal gross changes and toxic doses. Orally, slight or moderate decreased activity was observed at 500 mg./Kg., whereas toxicity was seen at 1,000 mg./Kg. The methiodide derived from fraction A was similar to that from fraction B. Intraperitoneally, curare-like effects occurred at 10 mg./Kg., whereas orally, toxicity was seen at 250 mg./Kg. Doses of 50 to 100 mg./Kg. failed to produce overt effects.

All of the samples caused consistent depression of respiration after treatment intravenously in the anesthetized (pentobarbital) cat. As in the dose range studies, the methiodides of the alkaloids were extremely toxic and caused respiratory embarrassment after doses as low as 1.0 or 2.5 mg./Kg. No significant hypotension or autonomic nervous system effects occurred without concurrent toxicity.

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Enteric Filmcoats by the Air-Suspension Coating Technique

By ROBERT E. SINGISER and WERNER LOWENTHAL

A rapid, efficient method of applying enteric filmcoats by a modified Wurster air-suspension technique is described. A cellulose acetate phthalate enteric filmcoating was applied to semiproduction size batches of ammonium chloride tablets. This method shows unique advantages over any coating method employing conventional coating pans. Smooth, uniform, pharmaceutically elegant enteric coatings are applied in a manner assuring passage of the U. S. P. enteric test. Monogramming on the tablets for identification remains easily identifiable under the filmcoat.

BECAUSE THE PRESENT method of enteric coating tablets gives inconsistent results and is time consuming, other methods have been proposed. Yen and Stirn (1) proposed dipping the tablets in the coating liquid. Neuhoff (2) recommended spraying the coating liquid onto the tumbling tablets in the pans. Spaulding (3) showed how to automate the coating pans. Clay and D'Angelo (4) described an automatic method of applying coating syrup and hot air to the tablets in coating pans. More recently, Tuerck and Carkhuff (5) described a method for spraying an enteric coating onto tablets tumbling in a pan. Wurster (6–8) invented a radically new method for coating tablets by suspending them in air and spraying the coating fluid onto the tablets continuously.

This paper describes a rapid and efficient method of applying an enteric filmcoat. This method shows distinct advantages over any method employing coating pans. The coating is applied utilizing the air-suspension technique (6–8) and a standard cellulose acetate phthalate (C.A.P.) solution to form the enteric coating. Accurately controlled amounts of enteric filmcoating can be uniformly applied. Passage of the U. S. P. XV test for enteric coated tablets is assured, eliminating the need for recoating and retesting.

Smoothness, color, and general pharmaceutical elegance can be achieved in the enteric filmcoating, eliminating the need for finishing the tablets with sugar coatings or other filmcoatings. Monogramming for corporate identification or dosage shows satisfactory definition through the enteric filmcoat.

The advantages of this method of application are: (*a*) smooth coating, (*b*) automated procedure requiring no skilled technicians, (*c*) no additional finishing overcoat required, (*d*) rapid application rate, (*e*) little or no loss of coating material on walls, etc., (*f*) no escape of solvent vapors into the work area, (*g*) tablets may be monogrammed, (*h*) no recoating required to assure passage of U. S. P. enteric coating test, (*i*) uniform amount of coating material applied to each tablet batch, (*j*) very little increase in tablet size, and (*k*) no dusting powder required.

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EXPERIMENTAL

Equipment.—An air-suspension coater similar to that described by Wurster (6–8) was used in this work. It was modified by constricting the orifice at the bottom of the coating chamber. This orifice was placed in the center of the chamber through a funnel-like adapter attached to the duct leading from the blower. Spraying of the filmcoat was done from the exact center of the orifice (see Fig. 1). In the apparatus described above, tablets are forced up the center of the column in a high velocity air stream. When the upward moving tablets reach the top of the main tablet bed they lose their momentum, fall back to the tablet bed, and move downward around the entire periphery of the column. This modification gives a different tablet circulation pattern than that seen in the Wurster apparatus (6–8).

Work was done in coating chambers of 6-, 10-, and 15-inch diameters, with respective capacities of about 4, 20, and 40 Kg. of tablets. The coating fluid was sprayed through a pneumatic atomizing nozzle made by Spraying Systems Co. (9).

Coating Solution.—Any C.A.P. solution now used for pan coating would probably be applicable, perhaps the formula will require dilution to spray properly.

Typical Formula for 1 Kg. of Tablets

C.A.P.	62.5 Gm.
Ethanol, absolute	240.0 Gm.
Plasticizer	10.0 Gm.
Dye	*q. s.*
Acetone	q. s. *ad* 1.0 L.

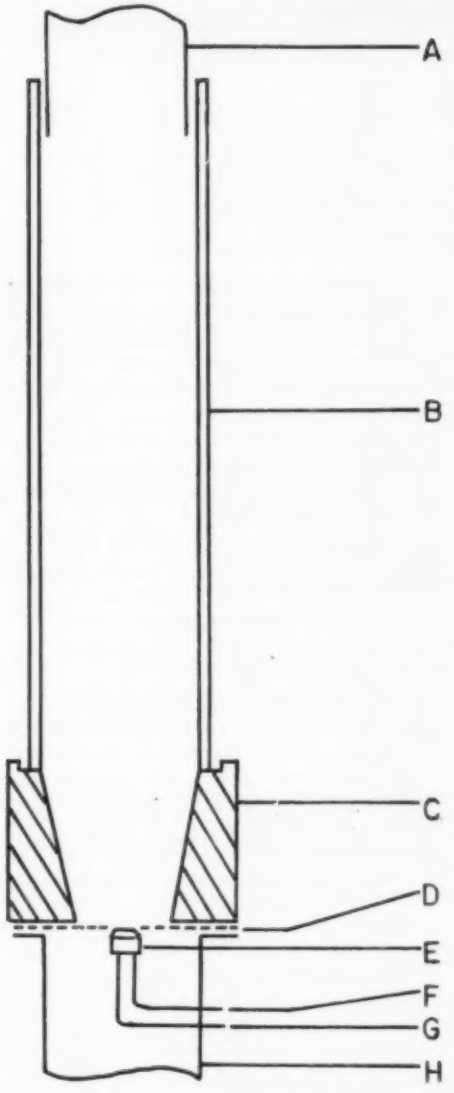

Fig. 1.—Schematic diagram of air-suspension coater. *A*, exhaust air duct; *B*, coating chamber; *C*, funnel-like modification; *D*, support screen; *E*, pneumatic atomizing nozzle; *F*, liquid feed for nozzle; *G*, air supply for nozzle; *H*, main air supply.

Dissolve the C.A.P. in 600 ml. acetone. Add the ethanol, plasticizer, and dissolve the dye with agitation. Add sufficient acetone to make 1.0 L. solution. Plasticizers such as propylene glycol, triacetin, or castor oil may be used. Surfactants may also be added if desired (10). If the enteric coat is not to be the final coat the dye can be deleted.

Technique.—The exact amount of enteric coating fluid needed for a given tablet load is put into the fluid reservoir. In these experiments 625 ml. of coating fluid per Kg. of tablets was used. Changes in the surface area of the tablet to be coated would require corresponding adjustment in the amount of fluid needed to obtain films of equal thickness.

The tablets to be used are loaded into the coating chamber. The air supply is turned on and the circulation pattern for the tablets is established. This should be a volume of air sufficient to keep the tablets freely circulating, but not so much as to cause tablet attrition. There is a reasonable range of air velocities that can be used. The inlet air is heated to 57–65°. As soon as the tablets are circulating the enteric coating fluid is introduced through the pneumatic atomizing nozzle.

Beyond this point the operator only has to control the flow rate of the liquid. This can be done by use of a metering pump or a needle valve in the liquid line. A thermometer in the exit air stream gives a good indication of the tablet bed condition. In these experiments the exit air temperature was maintained at 20–27°. The lower the exit temperature the faster the application of the enteric coating and the wetter the tablet surfaces. If the tablets become overwet the bed will clump together. Close regulation of the exit air temperature will prevent this and will give a smooth coating operation.

After the desired amount of enteric coating is applied, the tablet circulation is maintained until the exit temperature reaches 50°. The tablets are sufficiently dry to remove from the unit at an exit temperature of 32–38° but additional drying minimizes solvent retention. If engraved tablets are to be coated, a slower application rate may be required to prevent bridging of the film across the monogram depressions.

Once the conditions for proper operation are found, they need not be changed. No sticking of the coatings will occur.

On removal from the air-suspension coater, the tablets should be spread on trays and air or oven dried depending on the solvent-retention properties of the tablet cores and the solvent system used. In

Table I.—Ammonium Chloride Tablets Enteric Coated by Air-Suspension Technique

Run	Load Wt. Kg.	Coating Chamber Diam., in.	Coating Time, min.	Drying Time, min.	Enteric Passed, %	Test Time, min.
1	4.0	6	22 1/4	8	100	98
2	4.0	6	20 3/4	7 1/2	100	98
3	20.0	10	47 3/4	6	100	100
4	20.0	10	31 3/4	11	100	100
5	20.0	10	33 3/4	11	100	100
6	20.0	10	33 1/2	11	100	100
7	20.0	10	32 1/4	12	100	100
8	20.0	10	29	13	100	100
9	24.0	10	34 1/2	12	100	100
10	18.9	10	24	12	100	98
11	18.9	10	24	13	100	98
12	20.0	10	30 1/2	11 1/2	100	68[a]
13	30.0	15	38	15	100	70[a]
14	40.0	15	39	19	100	70[a]
15	4.0	6	20	8	100	70[a]

[a] Time in simulated gastric juice reduced from one and one-half hours to one hour.

TABLE II.—FILM THICKNESS

Tablet Size, in.	Shape	Tablet Surface Area, sq. in.	Tablet Weight mg.	Film Thickness			
				Face		Wall	
				Av. Thickness, mils[a]	Range, mils	Av. Thickness, mils[a]	Range, mils
13/32	Std. concave	0.441	500	4.20	3.50–5.25	3.81	3.00–4.50
13/32	Deep cup	0.402	500	5.35	4.50–6.25	4.27	3.25–5.25

[a] Average of 25 micrometer measurements.

addition, extremely fast coating rates may entrap solvent in the film and overnight drying would be desirable to allow the solvent to evaporate before bottling.

Table I lists the times required to complete various size representative runs of 500 mg. ammonium chloride tablets. The time required to make these runs compares most favorably with those reported by Tuerck and Carkhuff (5). They reported 50,000–75,000 tablets could be coated in one to two hours by spraying the enteric fluid onto tablets in coating pans. By this air-suspension method 80,000 tablets were coated in about forty minutes (run 14, Table I). Drying requires about twenty more minutes. If desirable, the drying could be done in warm air ovens and the tablets removed from the coating unit five minutes after the application of the enteric coat ceases.

The applied film thickness was determined from two coating runs. The amount of coating fluid applied per tablet was the same in each case, but the surface areas of the two tablets differed due to different shapes. Uniform coverage was obtained as evidenced by the enteric test results in Table I and the film thickness measurements in Table II.

Drying is almost instantaneous in the coating chamber, hence there is little danger of solvent penetration into the tablet core or solubilization of any of the tablet ingredients. The tablet roughness reported by Tuerck and Carkhuff (5) due to solubilization of tablet components was not found with this method.

CONCLUSIONS

1. A method of applying an enteric filmcoat has been developed to the semiproduction stage.
2. The time required for application of the enteric filmcoat compares favorably with those previously reported. This coat need not be followed by a finish coat.
3. Monogramming can be achieved for corporate identity without difficulty.
4. The finished tablet is only slightly larger than the uncoated tablet.
5. Passage of the U. S. P. enteric test is virtually assured, thus eliminating the need for recoating and subsequent retesting.

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Dehydrated Sterilizer Controls Containing Bacterial Spores and Culture Media

By JOHN H. BREWER and C. BAXTER McLAUGHLIN

A sterilization control of paper impregnated with spores of the heat-resistant organism, *Bacillus stearothermophilus*, and specific culture media in a dehydrated state is described. When suspended in distilled water, the media will elute from the test strip and support the growth of surviving bacteria. The necessity of preparing media for culturing the controls is eliminated.

From the time of Robert Koch (1) and his use of anthrax spores on a string, the most effective method of demonstrating sterilization has been the destruction of resistant bacteria of known characteristics.

In the pharmaceutical industry the increasing demands and requests for control of manufacturing processes may be aided by the use of a positive but harmless indicator containing viable heat-resistant bacteria.

In 1956, the authors (2) described a technique incorporating spores of the very heat-resistant *Bacillus stearothermophilus* in culture media contained in a sealed ampul to be used to check the effectiveness of steam-under-pressure sterilization. As a result of a continuation of our studies along this line, we would like to suggest a unique paper sterilizer control, different from those currently available. This control strip contains the same heat-resistant spores, plus a calculated amount of media sufficient to support growth of the organism when placed in water. With this indicator, which is designed for those occasions when it is impractical or undesirable to use the ampul type control, one places the strip in the autoclave load and, following the sterilization period, transfers it to a test tube containing 15 ml. of sterile distilled water and incubates at 55–65°. Media will elute from the strip and support the growth of any surviving bacteria.

Media of the following composition is used to impregnate the paper:

Trypticase peptone[1]	11.1%
Phytone peptone[1]	15.0%
Sodium chloride	1.5%
Bromcresol purple	0.066%
Agar	0.5%
pH 7.5	

Dissolve ingredients by heating, cool to 60°, inoculate with a suitable dilution of heat-resistant bacterial spores, impregnate paper, and air dry rapidly on a clean, flat surface. These papers are then cut into an appropriate size and are ready for use.

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[1] Baltimore Biological Laboratory, Inc., Baltimore, Md.

The aseptic use of this indicator is made possible by a specially designed envelope (3) that is readily opened to permit the removal of an inner envelope containing the test strip. This inner envelope is removed and, by inverting over a tube of sterile water, the impregnated strip is aseptically transferred.

If one is attempting to sterilize by steam under pressure a sterilizer load of "dry material"; i. e., surgical packs, gowns, dressings, etc., where the steam must penetrate considerable distances into the center, it would be advisable to use a control such as this strip. We have conclusively demonstrated by subjecting these paper controls to dry heat at 121° for twenty minutes that the bacteria are not killed. It is quite necessary to have moist heat at this temperature.

EXPERIMENTAL

Spore Preparation.—Spores are prepared by a slight modification of a technique we described in 1949 (4). It consists of a loop of Visking seamless cellulose tubing, $^{20}/_{32}$ inch inflated size, containing 50 ml. physiological saline suspended in 500 ml. of a medium of the following composition:

Trypticase peptone[1]	5 Gm.
Phytone peptone[1]	3 Gm.
Beef extract[1]	5 Gm.
Distilled water	1,000 ml.

The cellulose loop containing the saline is immersed in the medium and the entire assembly sterilized by autoclaving. On the following day, washings of *Bacillus stearothermophilus* from a trypticase soy agar slant are used to inoculate the loop. The assembly is incubated at 62–64° for eighteen to twenty-four hours. The spores are concentrated by centrifugation at 2,000 r. p. m. for twenty minutes and washed twice in distilled water. This low-speed centrifugation is rather differential and the majority of vegetative cells are removed. The residue is placed in boiling water for one hour to destroy the remaining vegetative organisms. With this method of preparing spore suspensions, heat resistance varied with each lot of spores prepared.

To count the viable spores, a strip is shredded in a small blender in 100 ml. sterile water, and, after soaking for thirty minutes, appropriate dilutions are made in distilled water containing 0.4% agar. Plate counts are prepared by first pouring 15 ml. of agar containing 0.37% trypticase, 0.5% phytone,

0.125% sodium chloride, 0.125% dextrose, 2.0% agar, 0.002% bromcresol purple, pH 7.5. A 1.0-ml. quantity of the semisolid agar dilution is then overlayed on the agar surface and spread evenly. This permits individual colonies to develop and reduces surface spreading to a minimum. Incubation is at 63–65° and colonies are readily discernible within twelve hours. Fermentation by the organism reduces the indicator and produces a yellow colony against a purple background.

Standardization and Evaluation.—The average autoclave is a relatively large cumbersome piece of equipment having many variables, and certainly not delicate enough for one to attempt to evaluate or standardize a control to be used for any autoclave. For this purpose we have constructed a pressure vessel heated by a thermostatically controlled electric heater, sealed with a pressure regulator, and containing a thermometer reaching the center of the container. Steam is generated within the vessel but excess moisture is prevented from reaching the articles under test by glass baffles. With this vessel we can rapidly handle the objects being sterilized, prevent excess heating, and cool immediately. For instance, we routinely standardize these control strips by using intervals of two and one-half minutes; i. e., strips will survive twelve and one-half minutes of 121° but not fifteen minutes.

A typical assay in which ten strips were tested at each time interval shows the following results:

	121° C.		
	10 min.	12½ min.	15 min.
Number of spore strips	10+	10+	10−

After eight to nine months' storage at room temperature, various lots of these sterilizer control strips show little, if any, loss in heat resistance. As they contain dehydrated media, storage in a moist atmosphere should be avoided.

Since each lot of spores are different in their heat resistance, counts are not routinely performed on the impregnated strips. Rather, appropriate dilutions are used, determined by trial and error. As an example, Lot No. 413 *B. stearothermophilus* spore suspension diluted as indicated produced the following results in regard to heat resistance and viable spores per strip before sterilizing.

A 200-ml. quantity of impregnation medium was inoculated with either (A) 30 ml. 1:100 dilution *B. stearothermophilus* spore suspension, lot No. 413, (B) 25 ml. 1:100 dilution lot No. 413, or (C) 20 ml. 1:100 dilution lot No. 413.

	121° C. Moist Heat				
Type	10 min.	12½ min.	15 min	17½ min.	Spores/Strip
A	10+	10+	10+	8+2−	29,200,000
B	10+	10+	10+	10−	25,800,000
C	10+	10+	1+9−	10−	19,500,000

Ten strips used for each type at each time interval.

More recent preparations successfully resist 121° for twelve and one-half minutes, having an approximate spore count of 1,400,000 viable spores per strip.

The use of this organism has been approved by the National Institutes of Health (5) as a sterilizer control for use in licensed establishments preparing biologicals because of its lack of pathogenicity, pyrogenicity, and toxicity. It is a thermophilic organism and will not grow at the official NIH and U. S. P. Sterility Test incubation temperatures (30–32°).

This spore-forming organism, *B. stearothermophilus*, NIH No. 7953, is rather unique; its optimum temperature of growth is 63–65° and it reaches maximum growth within six hours. Its entire metabolism is accelerated and therefore one may use these strips in an autoclave run and determine within twenty-four hours, and at the maximum, forty-eight hours, whether or not sterilization was achieved, rather than the usual impractical wait of seven days.

The use of an organism that requires such an elevated temperature of incubation is decidedly advantageous as the possibility of contamination due to technical error is reduced to practically nil. The rather small inconvenience of providing an elevated incubation temperature can be overcome by the use of a water bath at 60°, or a small incubator (6) is available that may be modified to provide this temperature.

In addition, an impregnated control has been successfully prepared, but containing a greater number of spores, to be used in dry heat sterilization at 160° for one hour. In a forced draft oven, after forty-five minutes the controls are positive; at sixty minutes, however, sterile conditions are indicated. We feel that if this control is used in the most difficultly-sterilized location within the oven, it will truly indicate the effectiveness of the procedure.

SUMMARY

A strip of paper may be impregnated with spores of *Bacillus stearothermophilus* and culture media containing an acid-base indicator to serve as a sterilizer control. After exposure it is merely necessary to place the control in a tube of sterile water and incubate. Media will elute from the strip and support the growth of surviving bacteria.

The proper adjustment of spores per test strip will yield a preparation sensitive to two and one-half minutes difference in autoclave sterilization time.

The organism suggested is nonpathogenic, nontoxic, and nonpyrogenic. It is esthetically acceptable as a bacterial control.

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Isolation of Oleanolic Acid and Saponin from Trefoil (*Lotus corniculatus*, var. *viking*)

By E. D. WALTER

Oleanolic acid was isolated from the alcohol extract of fresh trefoil (*Lotus corniculatus*, var. *viking*) and identified by comparison of its physical and chemical properties with authentic material. A small quantity of crude saponin was also isolated, which upon hydrolysis yielded soyasapogenol B.

IN AN INVESTIGATION of saponins in legume forages, the method used for the isolation of saponin from bur clover (1) and from ladino clover (2) was applied to fresh trefoil. When ether extraction was applied to the concentrated alcohol extract only a very small fraction of saponin appeared at the ether-water interface, but most of the material was soluble in ether. This indicated the presence of sapogenin rather than saponin, and that under the conditions of extraction it occurred uncombined. The melting point 304–305° of the crystallized material and the infrared spectrum were identical with those of authentic oleanolic acid.

This is another addition to the long list of plants that contain oleanolic acid either free or combined as saponin, which have been reported. Filtration of the solid material that appeared at the above mentioned water-ether interface yielded 22 Gm. of crude saponin, or about 0.06% of the dry weight of trefoil.

The low yield of saponin (water insoluble) and the occurrence of free oleanolic acid instead of saponin may account for nonexistence of bloat in ruminants from feeding on trefoil if it is assumed that saponins are one cause of bloat. No water-soluble saponin was obtained.

EXPERIMENTAL

Fresh trefoil (164 Kg.) was placed in a stainless steel tank and covered with 750 L. of 95% alcohol and allowed to stand for five days. The extract was drained and treated with charcoal (12 Gm. per L.) and filtered, with filter aid, in a filter press. The alcohol was distilled at atmospheric pressure until the volume remaining was about 90 L. Upon cooling white, microcrystalline material appeared. This was centrifuged in a continuous centrifuge. The solids were removed from the bowl and were suspended in water and again centrifuged and washed with water several times to remove water-soluble materials. The crude product was suspended in water and sufficient ether was added to dissolve most of the solids when shaken gently in a separatory funnel. A small quantity of insoluble material (saponin) appeared at the interface. The ether solution was clarified with charcoal and evaporated to near dryness. When recrystallized from methanol the product melted at 304–305°. Comparison with a known sample showed it to be oleanolic acid. Yield, 57 Gm. or about 0.17% of the dry weight of trefoil.

Soyasapogenol B.—The saponin was hydrolyzed by boiling for sixty hours in normal sulfuric acid in 65% ethanol. Water was added and the precipitated sapogenin was extracted with ether. Crystallization in methanol and chloroform gave a product melting at 260°. When mixed with soyasapogenol B the melting point was unchanged. The infrared spectrum was identical with that of known soyasapogenol B.

Acetyl Oleanolic Acid.—The oleanolic acid was acetylated with acetic anhydride. When recrystallized from ethanol and chloroform and dried at 120° in a vacuum it melted at 268°, $[\alpha]_D^{25} = +73.7°$.

Anal.—Calcd. for $C_{32}H_{50}O_4$: C, 77.06; H, 10.01. Found: C, 76.7; H, 9.89.

Methyl Acetyloleanolate.—The acetyl oleanolic acid (1Gm.) was dissolved in about 25 ml. of ether, and to this was added an ether solution containing an excess of diazomethane. The mixture was allowed to stand for twenty-four hours. Upon evaporation of the ether the product crystallized. Recrystallization in methanol and chloroform gave a product melting at 217–219°, $[\alpha]_D^{25} = +70.5°$ in chloroform. Frazier and Noller (3) reported the melting point 219–220° and $[\alpha]_D^{1*} = +70.4°$ for this compound.

Anal.—Calcd. for $C_{33}H_{52}O_4$: C, 77.29; H, 10.22. Found: C, 77.2; H, 10.0.

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Process for Pan Coating Small Batches of Tablets

By A. B. REDNICK, A. E. NICHOLSON, and S. J. TUCKER

A method is presented which permits the pan coating of small lots of tablets with production-size characteristics. This is readily accomplished by coating the tablets containing the active ingredient simultaneously with placebo tablets of the same dimensions. The procedure involves incorporating a small amount of reduced iron in the placebo, combining both the active and placebo tablets, coating the mixed batch of tablets and finally separating the active from placebo tablets by passing them through a magnetic selector.

THE ULTIMATE goal of the development pharmacist is to supply the manufacturing division with a tested formulation suitable for production. Unfortunately, during the development of a dosage form the pharmacist is sometimes confronted with a situation in which either there is a shortage of the chemical or the cost of the chemical is too great to warrant near commercial size lots in the development laboratory. As a result, it becomes necessary, from time to time, to coat tablets in very small lots, sometimes as few as 100 tablets.

The usual procedures, under these circumstances, have been to employ unrealistically small coating pans such as the top of a Silex coffee pot, or more commonly, to mix the active tablets with odd or different sized placebo tablets followed by sifting or garbling. The former method not only produces poorly coated tablets but also, most importantly, the results are not generally transferable to large scale operations. Even the latter method, although an improvement, usually gives rise to uneven coatings, doubles, and segregation in the pan because of size differential.

It has been found that the difficulties presently encountered in the coating of small batches of tablets, especially those with enteric coatings, can be overcome by modifying the procedure of adding odd size placebo tablets to the coating pan.

DISCUSSION

Generally speaking, the larger the pan load, the more efficient the coating process becomes and, what is most important, the more accurate and reproducible. It is also understood that when all the tablets are the same size and weight, the resulting product is more uniform and therefore more meaningful. Without having to sacrifice the chemical for development purposes, the following procedure was evolved which permits the achievement of a uniformly coated batch of tablets with the characteristics obtained in production size batches.

A production size batch of placebo tablets is prepared corresponding, in respect to ingredients, as closely as possible to the active tablet with the exception that a small amount of reduced iron is included. The placebo tablets are then compressed to the exact dimensional specifications of the tablets containing the active ingredient. The placebo tablets are then mixed with the active tablets and coated in the same pan. The finished mixture of tablets is then passed through a magnetic selector which efficiently separates the two products. As a result, the pharmacist has not only prepared a small batch of tablets with production-size characteristics at a minimum of cost, but has also prepared placebo tablets which could be used for clinical evaluation.

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EXPERIMENTAL

Approximately 20,000 calcium sulfate cores containing 5 mg. of reduced iron N. F. were prepared by the standard wet granulating technique. These cores were compressed using $^{5}/_{16}$ inch diameter punches having a radius of curvature of $^{5}/_{16}$ inch to give an average thickness of 0.122 inch and an average weight of 200 mg. This corresponded quite closely to cores which contained 5 mg. of prochlorperazine (as the dimaleate).[1] All these cores were then mixed and charged into a 14-inch stainless steel coating pan. The batch contained exactly 200 prochlorperazine cores. The coating procedure employed was that described by Tucker, Nicholson, and Engelbert (1).

The coated tablets, which were visibly indistinguishable except by breaking the tablets, were then passed through a magnetic selecting machine equipped with a Syntron vibratory elevator which served as a feeding device. (This equipment can handle about 30,000 tablets an hour; however, with modifications, amounts up to 70,000 an hour can be separated.) Exactly 200 tablets were recovered and were determined to be prochlorperazine tablets by destructive inspection.

This procedure was then repeated using 100,000 placebo tablets and 200 prochlorperazine tablets. Again, the 200 prochlorperazine tablets were recovered.

SUMMARY

1. A procedure is described which involves the simultaneous coating of tablets containing active ingredient and placebo tablets of the exact dimension but which contain a small amount of reduced iron. The finished tablets containing active ingredient are separated by a magnetic selecting machine.

2. The procedure allows large scale coating to be conducted with a minimum of chemical.

3. The procedure permits the production of placebos with the corresponding tablets containing the active ingredient at the same time.

4. The procedure permits accurate coating of small lots of enteric tablets.

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Determination of Piperazine Salts in Pharmaceutical Liquids

By R. E. PANKRATZ

A simple and accurate method is outlined for the determination of piperazine base and piperazine salts by a colorimetric method based on precipitation as the Reineckate. Control of pH is essential since acid solutions develop absorbance readings twice those of alkaline solutions.

THE WIDESPREAD use of piperazine salts as anthelmintics has caused considerable interest in analytical methods for their estimation.

The classic Kjeldahl total nitrogen method assay is suggested (1). This method is accurate but requires considerable time and manipulation.

Maynard (2) has used acetylation of piperazine combined with extraction as the basis of a gravinometric method or as an infrared spectrophotometric determination. Acetylation in an acetone solution is used by Bond (3). Both methods are time consuming in that they require filtration, drying, and weighing.

Cavett and Heotis (4) have modified a colorimetric method (5) which utilizes the color formed in the presence of quinone solution. Time and temperature are very critical during the color development in this method.

An excellent method for the determination of piperazine is titration in nonaqueous media such as glacial acetic acid. Glacial acetic acid made 0.1 *N* with perchloric acid gives an excellent end point with crystal violet or with glass and calomel electrodes. The interference of chlorides which may be present is easily eliminated by adding mercuric acetate (6).

When pharmaceutical preparations containing piperazine are available for analysis in dry, powdered form, nonaqueous titrations are very satisfactory, but nonaqueous titration can tolerate practically no water without introducing serious interference. This can be circumvented in liquid preparations by evaporating a suitable aliquot of the solution being assayed to dryness. This residue is redissolved in glacial acetic acid and titrated with standard perchloric acid. Very often the residue is of such consistency that it is necessary to warm it to bring it into solution. The author has encountered many instances of erratically low results due to partial acetylation of the piperazine. The use of acetic anhydride to eliminate the water present is not workable since piperazine readily acetylates in its presence.

Reinecke salt solution has been used by Kamath, *et al.* (7), to precipitate piperazine from pharmaceutical preparations when the pH was controlled between 9 and 10. Bandel (8) used the other extreme of the pH scale by adding citric acid to make the solution approximately pH 2. In both the above methods the piperazine Reineckate was dissolved in acetone and determined spectrophotometrically at 525 mμ.

The author has had the frustrating experience of getting widely divergent color intensities when the piperazine was precipitated as the Reineckate from a solution made slightly acid with dilute sulfuric acid (9). This inconsistency together with the widely divergent pH values used in the two successful methods suggested that the pH was critical in the quantitative precipitation of piperazine as the Reineckate.

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METHOD

Reagents.—Reinecke salt.

Reinecke solution was prepared by agitating 3 Gm. of the salt in 100 ml. water for fifteen minutes. This should be freshly prepared and filtered before each use.

Dilute Reinecke solution is three milliliters of the above diluted to one liter.

Standard piperazine solution was prepared by dissolving piperazine hydrochloride precisely equivalent to 0.2 Gm. piperazine in exactly 500 ml. water. The piperazine content of the salt was predetermined by the Kjeldahl total nitrogen method.

Buffer solutions were prepared at the pH values of 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 by the procedures of Clark and Lubs (10, 11). These were prepared at threefold strengths.

Sodium borate decahydrate.

Citric acid monohydrate.

Apparatus.—Conical 15-ml. centrifuge tubes. Clinical centrifuge or its equivalent.

All measurements were made with a Beckman DU spectrophotometer using 1-cm. cells.

EXPERIMENTAL

The standard curves were prepared by pipetting aliquots of suitably diluted standard piperazine to each of four centrifuge tubes. Each 5-ml. aliquot contained, respectively, 0.1, 0.2, 0.3, and 0.4 mg. of piperazine per ml. Five milliliters of the triple strength buffer solution were added to each tube, then 5 ml. freshly prepared Reinecke solution. The contents of the tubes were mixed thoroughly and allowed to stand for one hour in an ice water bath.

The tubes were centrifuged until the precipitate formed a solid cake. The supernatant liquid was carefully decanted and the precipitate was washed with 1 ml. ice cold dilute Reinecke solution. A small glass rod aided in loosening the cake and mixing.

All tubes were again centrifuged and the wash solution was carefully decanted. The tubes were allowed to stand inverted on a towel for five minutes. The precipitate was dissolved in exactly 10 ml. acetone and centrifuged lightly to clear the solution. All absorbances were determined at 525 mμ.

The series of standard curves were prepared using buffer solutions ranging from pH 1 to 10 at intervals of 1. These readings were plotted with the concentration as the ordinate and the absorbance as the abscissa. See Fig. 1.

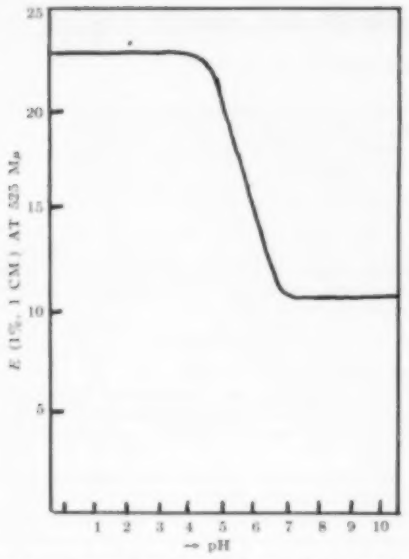

Fig. 1.—The extinction coefficient, $E(1\%,1\text{ cm.})$, of piperazine base as the Reineckate at 525 mμ *vs.* pH.

The reproducibility of the method was checked at two pH values. At pH 9.2 using 5 ml. saturated solution of sodium borate as the buffer and at pH 2 using 5 ml. 3% citric acid solution. The results are shown in Table I. The average deviation in both systems was within 1% which is often regarded as the limit of accuracy of colorimetric measurement.

Procedure for Liquid Preparations Containing Piperazine Salts or Base.—Dilute the sample solution in a volumetric flask in such a manner that it contains 0.5 mg. or more of piperazine base per ml. Pipet 5-ml. aliquots into each of two 15-ml. centrifuge tubes. To each, add 5 ml. 3% citric acid and 5 ml. Reinecke solution. Mix and allow to stand one hour in an ice water bath.

Centrifuge until the precipitate forms a firm cake and decant the supernatant liquid with care that no precipitate is lost. Wash the precipitate with 1 ml. dilute Reinecke solution. Use small rod to loosen the cake and to stir.

TABLE I.—ABSORBANCES AT 525 mμ FOR ALIQUOTS CONTAINING 2 MG. PIPERAZINE BASE

At pH 2		At pH 9.2	
Absorbance	Deviation	Absorbance	Deviation
0.470	−0.001	0.230	−0.004
0.465	−0.006	0.236	+0.002
0.470	−0.001	0.236	+0.002
0.475	+0.004	0.239	+0.005
0.460	−0.011	0.235	+0.001
0.471	0.000	0.237	+0.003
0.468	−0.003	0.236	+0.002
0.481	+0.010	0.231	−0.003
0.480	+0.009	0.232	−0.002
0.471	0.000	0.231	−0.003
Av. 0.471	0.004	0.234	0.003

Centrifuge again and decant with care. Allow the tubes to stand inverted on a towel for five minutes.

Dissolve the precipitate in exactly 10 ml. acetone. Centrifuge lightly to clear the solution and measure the absorbance at 525 mμ. The piperazine present can be found by referring to the previously prepared curve.

The author prefers to use an additional two centrifuge tubes each of which contain 5 ml. standard piperazine solution which represents 2 mg. base. These are treated in precisely the same manner and at the same time as the sample aliquots. The piperazine base in the sample can be calculated from the expression

$$0.0020 \times \frac{\text{absorbance sample}}{\text{absorbance standard}} \times \text{dilution factor} = \text{Gm. piperazine base per ml.}$$

DISCUSSION

Plotting the extinction coefficients, E (1%, 1 cm.), against pH shows a constant value from 1 through 4. The points from 5 and 6 are progressively lower, but at 7 through 10 the values again become constant. Since all standard curves cross the x and y axes at the point of intercept it can be safely assumed that the lower absorbances that occur at the higher pH values are not from increased solubility of the precipitate. See Fig. 2

Bandel (8) states that the piperazine Reineckate formed in the citric acid solution contains two molecules Reineckate to each piperazine molecule. An obvious explanation of the lower absorbances in the alkaline solutions is that the piperazine Reineckate has only one molecule Reineckate to each molecule piperazine. This is borne out in that the

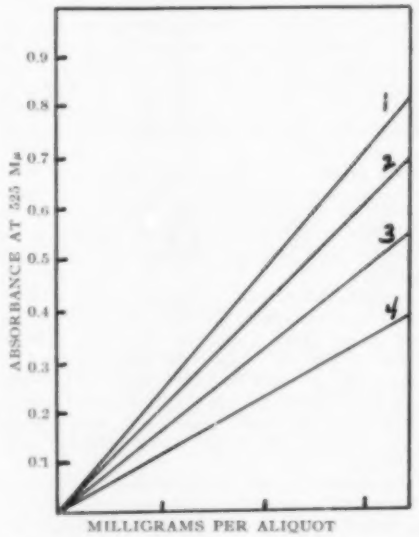

Fig. 2.—The standard curves of the absorbance *vs.* milligrams of piperazine base as the Reineckate per aliquot. 1, pH 1 through 4; 2, pH 5; 3, pH 6; 4, pH 7 through 10.

extinction coefficients at the acid levels are twice as great as those at alkaline values. The intermittent values for 5 and 6 are the result of different mixtures of the two forms.

Much difficulty was experienced in getting consistent results when the solutions were simply made slightly acid with dilute sulfuric acid instead of using a buffer solution of definite pH value. In the pH range from 4 to 7, slight differences in acidity made large differences in absorbances.

The reproducibility of the absorbance readings for the systems at 9.2 and 2 seems to be of the same order. Since the absorbance at the acid pH is twice as great, and the 3% citric acid solution is very easily prepared, using the acid pH is the method of choice.

The use of solutions at pH 9.2 is often of value since occasionally it is necessary to precipitate the piperazine in the presence of other quaternary ammonium salts which do not form Reineckates in the alkaline solution.

This method has been used in this laboratory for some time and has been completely satisfactory in routine quality control of piperazine solutions. The great advantage of this method is its simplicity and, since centrifuges and spectrophotometers are common equipment, it requires practically no special equipment.

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Technique for Studying Thermally Induced Phase Transitions

• By DAVIS R. REESE, PETER N. NORDBERG, STUART P. ERIKSEN†, and JOSEPH V. SWINTOSKY

AN APPARATUS has been designed to determine and record transition temperatures in crystalline materials. Polarized light transmission is measured as the sample is heated or cooled. Changes in crystal structure are indicated by changes in the light transmission. The technique detects any change in crystal structure except the rare isotropic crystal ⇌ isotropic liquid. This temperature-polarized light (Tempol) method is more versatile and more sensitive than the dilatometric and heating-cooling curve methods (1, 2), requires a minimum amount of sample, and permits changes to be photographed whenever they occur. The thermodynamic interpretations possible with the former methods, however, are not possible with the Tempol method as presently employed.

The Tempol apparatus is composed of six main components each of which are obtainable from a variety of sources: (*a*) a low power polarizing microscope, (*b*) a hot stage, (*c*) a controlling unit for the hot stage that produces linear rates of heating or cooling, (*d*) a photometer, (*e*) a recorder, and (*f*) a camera (optional).

The system depends upon the property of nearly all crystalline substances to resolve light into perpendicularly polarized beams (anisotropy). Placed between two crossed polarizing filters, these substances reorient the polarized light from the polarizer (lower filter) so that it can pass through the analyzer (upper filter). The fraction of light passed is dependent upon the angle between the optical axes of the crystals and the planes of the polarizing filters, the amount of material in the field, and the refractive indexes of the crystals.

Crystalline changes which alter the refractive indexes or the crystal habit of a solid may be detected by observing the changes in light intensity transmitted by the sample when viewed with a polarizing microscope. Similar changes are also observed with liquid ⇌ crystalline phase changes. Temperature affects both refraction and crystal habit, so that polymorphic transition temperatures, phase change temperatures, and solvation changes can be observed by monitoring polarized light transmission while the sample is being heated or cooled.

The Tempol apparatus has one or more advantages over previous designs (3–5) in that a permanent record is obtained of all transitions occurring throughout the temperature range studied and, because the rate of temperature change is constant, all crystalline transitions occur through a constant thermal gradient. Two curves are more readily compared if their heating rates are identical. Furthermore, the apparatus design permits visual inspection of the sample through the second eyepiece and simultaneous use of a camera without interrupting the recording process.

Subtle structural changes in crystals may at times

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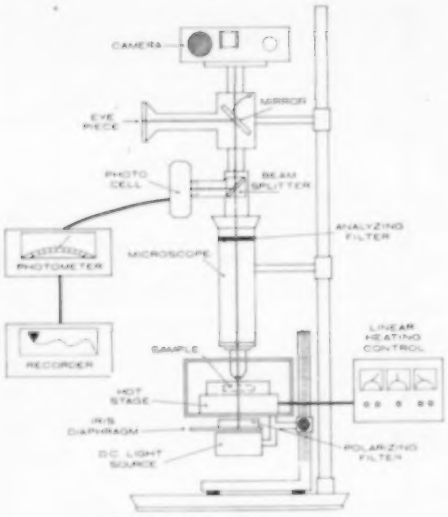

Fig. 1.—Tempol apparatus.

be detected by optical methods when they are undetectable by the usual thermal and dilatometric methods. A number of changes have been detected by the Tempol apparatus which are not identifiable from the literature, even on pure materials. In addition, the sample size using this method may be reduced to that needed to fill the field of the microscope (less than 5 mg.).

Figure 1 is a schematic drawing of the apparatus. At the bottom of the figure are the hot stage (a Leitz model 350, insulated with magnesia block) and light source (an American Optical universal microscope illuminator operated from a 6 v. automobile battery). These are attached by a common link to a rack and pinion movement. The remaining items in the light train are in fixed positions. Direct current is desirable in the light source since minute voltage changes cause significant fluctuations on the recorded curve. The hot stage is insulated to enhance precise temperature control. The linear heating control unit is a thyratron thermoregulator circuit using a resistance thermometer mounted in the hot stage as the detection element and a motor driven potentiometer as the reference element in a Wheatstone bridge circuit. The exact circuit design will be published elsewhere.

Light passes through the sample, then through the microscope, and finally enters the beam splitter. Part of the light entering the beam splitter is reflected to the photocell. This signal is amplified by the photometer (a Photovolt model 501-M) and continuously recorded on a Brown electronic potentiometric recorder.[1] The remainder of the light passes through the beam splitter and is reflected by a mirror into the eyepiece. To take a photomicrograph, the mirror is swung into a vertical position and the camera shutter is opened. If photomicrographs are not desired, the photocell is mounted above the beam splitter and the eyepiece replaces the photocell in the diagram.

[1] While *x-y* recording might seem desirable, the linearity of the heating rate makes the added cost unnecessary.

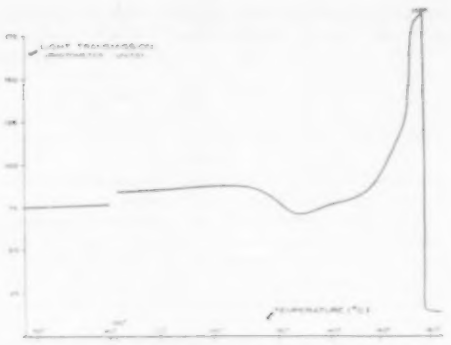

Fig. 2.—Tempol curve of amobarbital U. S. P.

The use of the Tempol technique for studying crystal transitions may be illustrated with data for amobarbital U. S. P. A mounted microscope slide of amobarbital, previously recrystallized from ether, was prepared by allowing molten drug to flow into a thin chamber formed by separating a microscope slide and a cover glass with two narrow shims made from cover glass stock. The molten film was allowed to cool slowly to room temperature and placed inside the hot stage. Polarized light of constant intensity was passed through the mounted sample and the photometer-recorder system adjusted. The sample was then heated at two degrees per minute, starting at room temperature and continuing until melting was complete. The light transmission curve is shown in Fig. 2. The minimum in the curve at approximately 134° (uncorrected) indicates that amobarbital II has transformed into amobarbital I. This change was also observed visually and is in the range for a similar change observed by Cleverly and Williams (6). The gradual increase in light transmission between 134 and 158° indicates further transformation in amobarbital prior to melting. This increase in light transmission could indicate: (a) disappearance of crystal dislocations prior to liquefying, (*b*) formation of a new crystal form, or (*c*) premelting which might reduce light scattering. The melting point is indicated by the peak at 158°. Complete melting of amobarbital, as indicated by the onset of the horizontal section near the end of the curve, occurs at 158.8°.

The Tempol technique promises to be a valuable method for detecting and recording crystal changes that result because of preparative procedure, temperature, pressure, and other variables. The technique can be used, for instance, to obtain melting or solidification points, to study loss of solvent of crystallization, and to study the effect of tempering a polymorphic substance.

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Purification of Dimethylformamide for Use in Nonaqueous Titrations

By R. E. MOSKALYK, L. G. CHATTEN, and M. PERNAROWSKI

DIMETHYLFORMAMIDE (DMF) has proved to be a most useful solvent for the nonaqueous titration of many organic acids. Its major advantage is that it is a relatively weak base and hence does not absorb carbon dioxide as readily as do other more basic solvents. Its major disadvantage is its susceptibility to hydrolysis in the presence of water. Removal of all traces of water from DMF is a very difficult process (1) and consequently commercial DMF invariably contains acidic impurities among which are water and formic acid. These impurities are usually present in small quantities and are compensated for by neutralization of the solvent immediately prior to its use or by a determination of the quantity of titrant consumed by a specified quantity of DMF. Excessive impurities, however, can result in interfering side reactions and, therefore, in these cases the solvent must be purified prior to its use.

Thomas and Rochow (1) reported four methods for the removal of proton-releasing impurities such as water from DMF. These methods all involve chemical separation followed by distillation under reduced pressure. One of these methods, involving periodic shaking with barium oxide prior to distillation, has been found to be suitable for the purification of DMF when it is to be used for nonaqueous titrations (2). Brown (3) removed formic acid from DMF prior to distillation by precipitating it as ammonium formate. Since DMF decomposes slightly at its normal boiling point to give dimethylamine and carbon monoxide, distillation must be carried out at reduced pressure. However, this decomposition is catalyzed by the presence of acidic or basic impurities and, hence, could occur at lower temperatures.

We have used a method of purification which is simple and effective and does not require redistillation of the solvent. The commercially available DMF which we had at our disposal yielded a blank titration equivalent to 0.4 cc. of 0.1 *N* methanolic potassium hydroxide per 50 cc. of the solvent. After purification of the solvent, by the method described below, the blank was reduced to 0.05 cc. of 0.1 *N* methanolic potassium hydroxide per 50 cc. of DMF.

PROCEDURE

Fifty grams of Dowex 1-X10, 100–200 mesh, was converted to the hydroxyl form by stirring the resin with 10% sodium hydroxide solution for several hours. The regenerated resin was washed with distilled water until the washings were neutral to phenolphthalein, then with methanol, and finally air dried. The dry resin was placed in a large Erlenmeyer flask fitted with a glass stopper, 500 cc. of DMF was added, and the mixture stirred for several hours. The DMF was then decanted, filtered, and the blank determined on 50 cc. of the solvent. This process could be repeated several times before regeneration of the resin was required.

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(1) Thomas, A. B., and Rochow, E. G., *J. Am. Chem. Soc.*, **79**, 1843(1957).
(2) Allen, J., and Geddes, R. T., *J. Pharm. and Pharmacol.*, **9**, 990(1957).
(3) Brown, D. J., *J. Appl. Chem.*, **1**, Suppl. Issue **2**, 159(1951).

Book Notices

Laboratory Manual of Medical Microbiology. By JANET B. GUNNISON. N-P Publications, 850 Hansen Way, Palo Alto, Calif., 1960. viii + 243 pp. 14 × 21.5 cm. Paperbound. Price $3.50.

A manual designed as a guide to laboratory work in microbiology for students of medicine, dentistry, and pharmacy, the book is intended for use as a reference along with mimeographed assignments on daily schedules. The teaching approach is interesting. An index is included.

Organic Reactions. Vol. 11. Edited by ARTHUR C. COPE. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1960. vii + 501 pp. 15 × 23 cm. Price $12.

Material covered in the eleventh volume of this important series includes: The Beckmann rearrangements, The Demjanov and Tiffeneau-Demjanov ring expansions, Arylation of unsaturated compounds by diazonium salts, The Favorskii rearrangement of haloketones, and Olefins from amines: the Hofmann elimination reaction and amine oxide pyrolysis. A subject index for this volume and cumulative chapter and author indexes for volumes 1–11 are appended.

Drugs and Behavior. Edited by LEONARD UHR and JAMES G. MILLER. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1960. xix + 676 pp. 15 × 22.5 cm. Price $10.75.

The text material of this book includes 51 chapters contributed by individuals or joint authors, and is divided into two parts under the headings: The methodological, chemical, biological, and clinical context for psychopharmacology and Experimental procedures and results. Methods of study of the effects of psychoactive drugs in human subjects are discussed with particular emphasis on the full spectrum of therapeutic and psychosomatic results and behavioral toxicity of tranquilizers, energizers, psychotomimetics, etc. Although the book is designed primarily to aid the general practitioner of medicine, it is a useful reference for the pharmacist and others interested in drugs and therapeutics. An excellent subject index and an author index are included.

Accepted Dental Remedies 1961. 26th ed. Council on Dental Therapeutics of the American Dental Association. American Dental Association, 222 E. Superior St., Chicago 11, Ill., 1961. xiv + 230 pp. 15.5 × 20.5 cm. Paperbound. Price $3.

The principle function of Accepted Dental Remedies (ADR) is to provide the practicing dentist with information on the usefulness of drugs in dentistry. The 1961 edition is well designed for this purpose and for use as a handbook of dental therapeutics for students as well as practitioners. The expanded sections on dental therapeutics alert the dentist to special problems that may be encountered when a patient seeks dental advice while receiving medication or treatment by a physician, particularly when the medication consists of potent drugs such as corticoids, or in case histories of diabetes or cardiovascular disease.

The chapter on "Fluoride Compounds" includes a discussion on dentifrices containing stannous fluoride and explains why the toothpaste Crest has been accepted for inclusion in Group B. The book includes provisions for acceptance of products, a general index, a distributors' index, an index of current reports from the Council on Dental Therapeutics, and an index of more recent reports on products not listed in this edition, including those classified in Groups B, C, and D.

A brief section on Formulas and Tables should be of particular interest to pharmacists, and they will find ADR a useful reference source.

Theoretical and Practical Problems of Medicine and Biology in Experiments on Monkeys. Edited by I. A. UTKIN. Pergamon Press, Inc., 122 East 55th St., New York 22, N. Y., 1960. vii + 276 pp. 14 × 21.5 cm. Price $9.

Investigations of physiological and pathological problems in experiments on monkeys at the Institute for Experimental Pathology and Therapeutics, USSR Academy of Medical Science, are reported in this book (translated from the Russian). The text material is divided under the three main headings: Physiology and pathology of higher nervous activity, Pathological morphology and physiology, and Infectious pathology. An index is appended.

Handbook of Physiology. Section 1: Neurophysiology. Vols. 1, 2, and 3. Edited by JOHN FIELD. American Physiological Society, Washington, D. C., Vol. 1, 1959; Vols. 2 and 3, 1960. Distributed by The Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md. Vol. 1, 780 pp.; Vol. 2, 660 pp.; Vol. 3, 573 pp. 21.5 × 28 cm. Price: Vol. 1, $22; Vols. 2 and 3, $20.

While the multi-volume Handbook of Physiology will afford a current view, it is the publisher's intent to present the various facets of the subject in due proportion to their importance. It will not, therefore, be devoted unduly to recent advances in the field but will cover discoveries and formulations regardless of the time they were made. Specialists in specific phases are contributors to this compilation. Separate subject indexes are included in vols. 1 and 2, and composite author and subject indexes for the 3 vols. are appended in vol. 3. Under the index entry "Historical development" the subdivision "contributors" gives an alphabetical listing. This type of listing would be useful in books on other sciences. The series is an excellent addition to the medical reference library.

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